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The regulation of tyrosine phosphorylation during the differentiation of a human leukemia

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THE REGULATION OF TYROSINE PHOSPHORYLATION DURING THE DIFFERENTIATION OF A HUMAN LEUKEMIA

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ABSTRACT

THE REGULATION OF TYROSINE PHOSPHORYLATION
DURING THE DIFFERENTIATION OF A HUMAN LEUKEMIA

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The development of neoplastic cells may be viewed as a defect in the normal differentiation process of these cells. All proliferating tissue is in a homeostatic balance between proliferating immortal stem cells and non-proliferating, functionally mature terminally differentiated cells. A block in the process of maturation leads to a buildup of dividing undifferentiated cells and the formation of a malignancy. As such, the study of differentiation can shed light on the process of normal cellular regulation, the genesis of tumors, and, perhaps, strategies for combatting neoplasia. HL-60 promyelocytic leukemia cells are an excellent model system for studying hematopoietic differentiation. These cells grow as promyelocytes in tissue culture, but they can be induced to mature into granulocytes (by agents such as dimethyl sulfoxide, retinoic acid, and some anthracycline antibiotics) or into monocytes (by agents such as phorbol esters). In studying this system, it was desired to examine a biochemical

process that would be a central mediator of the control of proliferation and differentiation. Work in viral systems has shown that the phosphorylation of tyrosine residues in proteins is intimately associated with cellular transformation. Furthermore, a number of growth factor receptors have been shown to possess tyrosine kinase activity which is essential for their ability to induce a proliferative response. As such, the present study sought to deterimine how tyrosine phosphorylation and the enzymes which regulate it are controlled during HL-60 differentiation.

HL-60 cells normally contain about 1.5% of their phosphoaminoacids as phosphotyrosine. Upon granulocytic differentiation, phosphotyrosine content decreases to 0.1 to 0.3% of phosphoaminoacids. HL-60 cells possess a tyrosine kinase activity located in the particulate fraction which can phosphorylate a number of tyrosine-containing substrates in a time- and temperature-dependent fashion. It has a k_m for ATP of about 20 uM, a pH optimum of 6.4, and a preference for Mn^{2+} (12 mM) as a co-factor; it is further stimulated by Zn^{2+}, does not respond to insulin or epidermal growth factor, is activated by detergents, and is inactivated by N-ethyl-maleimide. These cells, in addition, possess a phosphotyrosine phosphatase activity which is also located in the particulate fraction, dephosphorylates phosphotyrosine in a time- and temperature-dependent manner, is inhibited by Zn^{2+} and VO_4^{3-}, has a pH optimum of 7 to 8,

and seems distinct from alkaline and acid phosphatases. This activity is similar to, if not identical with, p-nitrophenyl-phosphate hydrolysis activity. Both the kinase and the phosphatase appear as single peaks through preliminary purification.

During the granulocytic differentiation of HL-60, there is a 2.5-to 3.5-fold increase in tyrosine kinase activity, and a 5- to 8-fold increase in phosphotyrosine phosphatase activity. Two cell lines resistant to dimethyl sulfoxide-induced differentiation were derived and employed to show that these changes were differentiation-specific and not non-specific drug effects. Several anthracycline antibiotics and a hypoxanthine-guanine phosphoribosyl tranferase-deficient subline sensitive to induction of differentiation by 6-thioguanine were used to demonstrate that these changes were not secondary to growth inhibition, but were due to differentiation per se. A murine myelomonocytic leukemia, WEHI-3B, was also studied and found to display similar changes in phosphotyrosine and tyrosine kinase and phosphotyrosine phosphatese activities with differentiation.

Monocytic differentiation of HL-60 cells was found to be accompanied by an order of magnitude decrease in phosphotyrosine residues, a 2-fold increase in tyrosine kinase activity, and a 10-fold increase in phosphotyrosine phosphatase activity.

Thus, both granulocytic and monocytic differentiation of HL-60 cells are accompanied by a large fall in total phosphotyrosine, an increase in tyrosine kinase activity, and a still larger increase in phosphotyrosine phosphatase activity. This system represents an excellent model for dissecting the importance of phosphotyrosine regulation in normal differentiation and the development of neoplasms, and suggests possible novel approaches for the treatment of malignancies.

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LIST OF ABBREVIATIONS

ACM	aclacinomycin A
ADR	adriamycin
AEV	avian erythroblastosis virus
ASV	avian sarcoma virus
cAMP	cyclic adenosine monophosphate
CSF-I	colony stimulating factor-I
DEAE	diethyl aminoethyl
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
env	retroviral envelope gene
gag	retroviral group specific antigen gene
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid
HGPRT	hypoxanthine-guanine phosphoribosyl transferase
MCM	marcellomycin
MES	2-(N-morpholino)ethane sulfonic acid
MOPS	3-(N-morpholino)propane sulfonic acid
MW	molecular weight
NBT	nitroblue tetrazolium
NEM	N-ethylmaleimide
NGF	nerve growth factor
NP-40	nonidet P-40
NTE	0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA
OAG	1-oleoyl-2-acetyl-glycerol
P_i	inorganic phosphate
PAGE	polyacrylamide gel electrophoresis
PDGF	platelet-derived growth factor
PNPP	p-nitrophenylphosphate
pol	retroviral RNA-directed DNA polymerase (reverse transcriptase) gene
pp60,pp60^{src}	60,000 molecular weight tyrosine kinase originally isolated from retroviruses
P-Ser	phosphoserine
P-Thr	phosphothreonine
P-Tyr	phosphotyrosine
RA	retinoic acid
RNase	ribonuclease
RSV	Rous sarcoma virus
SDS	sodium dodecyl sulfate
SSV	simian sarcoma virus
TCA	trichloroacetic acid
td	transformation defective
TG	6-thioguanine
TLCK	Na-p-tosyl-L-lysine chloromethyl ketone
TPA	12-O-tetradecanoyl phorbol 13-acetate
ts	temperature sensitive

INTRODUCTION

The study of cancer and the study of cellular differentiation and proliferation are intimately related, as the former may frequently be an aberration of the latter. Thus in trying to understand cancer, and in designing chemotherapeutic agents which can specifically inhibit malignant cells, an understanding of normal cellular regulatory functions is critical. During the processes of proliferation and differentiation, changes occur in numerous intracellular reactions; yet it is likely that only a few of these changes are central to the process, the remainder only sequelae. The first clues as to what those central control processes are has come from viral systems.

Tyrosine phosphorylation in retroviral systems. The acute transforming retroviruses are a subset of the retrovirus family. Retroviruses contain RNA as their genetic material, and can propagate without causing cell death. Their life cycle is unique in that upon entering cells, their RNA is "reverse transcribed" into DNA which then integrates into the host genome and directs the synthesis of viral-specific RNAs. The retrovirus genome normally codes for three genes. Of these, two are structured: env (for the envelope protein), and gag (for group specific antigens); and one is enzymatic: pol (for RNA-directed DNA polymerase -- reverse transcriptase). The acute transforming

retroviruses contain a fourth gene, the so-called oncogene, which gives them their distinctive ability to rapidly cause tumors when injected into animals, and to cause the malignant transformation of cells in tissue culture. The prototypic member of this family, on which the seminal work on oncogenes was performed, is the Rous Sarcoma Virus (RSV; reviewed in reference 1a).

When mouse 3T3 fibroblasts are grown in tissue culture, they form a monolayer, one cell thick, then stop growing -- a phenomenon known as contact inhibition. Although these cells are not "normal" (as are primary fibroblasts) in that they will grow indefinitely in tissue culture (immortality), they are not neoplastically transformed either. When these cells are exposed to RSV, the monolayer is speckled with grossly observable foci. These foci differ phenotypically in numerous ways from the parent 3T3 cells: they display an absence of contact inhibition, growing in multiple poorly defined layers (hence the macroscopically visible foci); they exhibit morphological changes, appearing rounded and spindly compared to the more elongated non-transformed cell; they manifest a decreased serum dependence for growth; they obtain a greater proportion of their energy from glycolysis; and finally, the sine quo non of malignant transformation, they will form tumors when injected into nude (athymic) mice.

Genetic analysis provided the clue that a distinct

gene product was responsible for transforming ability. A number of mutants of RSV were derived that either lacked the ability to transform cells (transformation defective or td), or were temperature sensitive (ts) in transforming ability (1b, 1c). Both classes of mutants, however, displayed normal replicative properties; this suggested that a separate non-structural non-essential gene, designated *src* (for sarcoma), was responsible for transformation. That the product of a single gene (an oncogene) could cause the myriad changes associated with neoplastic transformation was a startling concept, and led to an active search for the product of that gene. Two fundamental approaches were used to isolate the *src* protein (1). The first involved immunoprecipitating proteins from cells transformed by avian sarcoma virus (ASV, closely related to RSV), using antisera prepared from rabbits bearing ASV-induced tumors. It had been shown in several other tumor virus systems (SV40, polyoma, adenovirus) that animals carrying these neoplasms develop antibodies to nonstructural virus-encoded polypeptides. The precipitated proteins could then be compared to those precipitated from cells infected with td strains; the polypeptide(s) unique to the transformed cells would likely represent the product of the *src* gene. The second approach, employing similar logic, involved the use of *in vitro* translation of mRNA. A comparison of the proteins translated from the RNA of either transformation

competent or td viruses would be expected to yield information on the transformation-specific polypeptide. Purchio et al (1) employed both of these techniques, and found that each identified a single 60,000 molecular weight (MW) protein unique to the transforming strain. Proteolytic digestion techniques suggested that the proteins identified by each method were identical.

Given this strong evidence for the relationship of the 60,000 MW protein with transformation, a number of groups began to further characterize this product. Brugge and coworkers (2) demonstrated that apparently identical 60,000 MW proteins were found in the cells of three different species (chick, field vole, and hamster) transformed by ASV. This supported the hypothesis that the protein was in fact of viral origin, and not a cellular protein induced by ASV. Levinson et al. (3) showed that this protein was phosphorylated, and dubbed it pp60 (or $pp60^{src}$). These workers also found that temperature-sensitive mutants of the src gene were able to produce equal amounts of the protein as the parent, but the phosphorylation of pp60 was decreased at the non-permissive temperature. This suggested that phosphorylation of pp60 might be critical for its function. They further demonstrated that pp60 contained protein kinase activity, and could transfer the terminal phosphate of either ATP or GTP onto immunoglobulin molecules in immunoprecipitates. This activity was also found to be

temperature sensitive in the mutants, and suggested a mechanism whereby this protein could effect transformation. Collett and Erikson (4), in similar work done at that time, also suggested that protein phosphorylation might be a mediator of transformation.

Protein kinase activity was an attractive enzymatic function for a transforming protein, in that it could explain how a single gene product could effect the numerous changes seen in malignant cells. Erikson et al. (5) began the characterization of the enzyme by demonstrating that the protein kinase activity was cyclic AMP (cAMP)-independent. They further showed that not only can pp60 phosphorylate immunoglobulins in immunoprecipitates, but it can also phosphorylate exogenously added casein.

The most dramatic insight into the function of the *src* gene product came in experiments performed by Hunter and Sefton (6). A number of protein kinases had been known at that time, and it was not clear what distinguished pp60, or what gave it the ability to transform cells. Hunter and Sefton found that this protein had the ability to phosphorylate tyrosine residues in proteins, an activity which had never been previously reported.

Although they demonstrated tyrosine phosphorylation in an *in vitro* kinase assay in immunoprecipitates, they presented three pieces of evidence supporting the view that pp60-mediated tyrosine phosphorylation is important *in vivo*

as well. First, following transformation by RSV, cells contain eight times as much total phosphotyrosine in proteins. Second, of the two phosphorylation sites on pp60 itself, one was found to be a tyrosine phosphorylation (the other being on serine). Finally, a 50,000 dalton phosphoprotein that could be coprecipitated with pp60 in RSV transformed cells also contained phosphotyrosine. The relationship of tyrosine phosphorylation to viral transformation was of great impact; yet of even broader significance was the understanding of the role of tyrosine kinases in normal cellular growth and differentiation, and in non-viral tumorigenesis.

In 1978, Spector and co-workers (7) found that sequences related to the src gene were found in the genomic DNA of a number of uninfected vertebrates. Using DNA annealing techniques, they found homologous segments in cells from chickens, humans, calves, mice, and salmon, but not in sea urchin, Drosophilla, or E.coli. In contrast, only chicken DNA contained sequences homologous to the remainder of the ASV genome. Furthermore, thermal denaturation experiments showed that these sequences were highly conserved among these species. These data suggested that the src gene might code for a protein performing some critical function in cellular regulation. One year later, immunoprecipitation using antibodies to pp60, was used to show that various normal cells contained a 60,000 dalton

phosphoprotein which also contained protein kinase activity (8). Thus, uninfected frog, chicken, rat, and human cells but not Drosophila cells contain such a protein. Peptide maps of these proteins indicated that they all had major similarities to the src protein, but were not identical to it. This further suggested that not only are the genomic src sequences highly conserved, but that a protein quite similar to pp60 is produced in these diverse cells. That the product had protein kinase activity also supported the postulated important role it played.

All of the evidence implicating the cellular src sequences in the regulation of growth control led to a theory of the formation of acute transforming retroviruses. It was known that following the conversion of the retoviral RNA to DNA (by reverse transcriptase), the viral DNA integrates into the host genome. If in the process of integration the viral genome becomes associated with a cellular gene such as src, it is possible that in the formation of RNA for the reproduction of the virus, the cellular src gene may become part of the viral RNA, and be incorporated into new viral particles. The incorporation of the cellular gene may be at the expense of one of the genes for a viral protein, in which case the hybrid virus will be replication defective, and will need a helper virus to reproduce (as is the case with most transforming viruses). On the other hand, the src gene may be incorporated without

the loss of critical viral sequences, in which case the resultant virus will be replication competent (as with RSV). Strong support of this hypothesis was presented by Wang and co-workers (9) who demonstrated that transformation defective ASV strains could become transformation competent by a recombination event between the defective viral src gene and the normal cellular sequence. Presumably, then, once the src gene comes under control of the viral genome, its inappropriate expression will lead to transformation. This atypical expression may be either from increased production, inappropriate cellular localization, inappropriate expression in relation to the cell cycle, accumulation of mutations in the gene, or some other process.

Hunter and Sefton (6) found that the normal cellular src protein also contained tyrosine kinase activity. This was further evidence of the functional similarity between the normal protein and the viral product. Furthermore, the implication from this work that all normal vertebrate cells contain at least one highly conserved tyrosine-specific protein kinase, and that such a kinase acting alone in a virus can cause transformation, suggested that tyrosine phosphorylation may be a critical mediator of cellular growth control.

Since the seminal work on the src gene, several other viral oncogenes have been found to possess tyrosine kinase activity. These include fps (found in the Fujinami sarcoma

virus of chickens), fes (found in the Synder-Theilen feline sarcoma virus), yes (found in the Y73 chicken sarcoma virus), ros (found in the UR2 chicken sarcoma virus), and abl (found in the Abelson murine leukemia virus, which causes pre-B lymphomas) (10). Any doubt that the viral proteins themselves possessed the tyrosine kinase activity (as opposed to being co-purified with a cellular kinase) was dispelled when the src (11) gene was expressed in E.coli using recominant DNA technology. The onc proteins expressed in the bacteria (which lack tyrosine kinase activity) possessed full activity.

Growth factor receptor tyrosine kinases. With all of this evidence pointing to the importance of tyrosine phosphorylation in transformation, and with the findings that these viral enzymes most likely arose from the host genome, the question arose as to the function of these proteins in eukaryotic cells. At about the same time as the activity of these viral oncogenes was being elucidated, work was being done on dissecting the mechanism of signal transduction for growth hormone receptors. Cohen and his co-workers (12) were studying the activity of the epidermal growth factor (EGF) receptor in A431 human epidermoid carcinoma cells. They found that the binding of EGF to its purified receptor led to the phosphorylation of the receptor itself, as well as a number of other endogenous membrane proteins on tyrosine residues (13). It was subsequently

shown by others that treatment of A431 cells with EGF led to a five-fold increase in cellular phosphotyrosine content (14).

Following these studies with EGF, it was shown that the receptors for other polypeptide growth factors -- including insulin (15), insulin-like growth factor-I (16), platelet-derived growth factor (PDGF) (17), and colony stimulating factor-I (CSF-I) (18) -- also possessed tyrosine kinase activity that was stimulated by ligand binding. The relationship between the viral tyrosine kinase and the host proteins now appeared more clear. Normal cells possess receptors which allow them to respond to a variety of growth factors. The binding of the specific ligand leads to receptor activation which allows expression of tyrosine kinase activity. Phosphorylation of specific proteins on tyrosine leads to a proliferative response; upon removal of the growth factor, the receptor returns to its basal, unactivated state. Protein phophotyrosine phosphatases would then cleave the phosphate group from the tyrosine, allowing the cell to return to quiescence. The retroviruses, by incorporating these normal tyrosine kinases into their RNA genome would cause transformation by allowing these kinases to be expressed inappropriately. Two variations of this scheme have been elucidated for viral oncogenes. The first is typified by the erb-B gene (19) (as well as the fms gene (18)), one of the oncogenes of the

avian erythroblastosis virus. The erb-B gene codes for a protein which is a truncated version of the EGF receptor, that possesses tyrosine kinase activity yet lacks the EGF binding domain. Presumably, then, this enzyme, which is activated independently of the presence of the growth factor, leads to uncontrolled tyrosine phosphorylation and transformation. The *fms* oncogene, which is related to the CSF-I receptor, likely functions in a similar fashion (18).

The second mechanism of transformation related to growth factor receptors is exemplified by the oncogene of the simian sarcoma virus (ssv). The product of this *sis* gene is very closely related to PDGF (20); although it is probably not secreted from ssv-transformed cells (21), it can interact with PDGF-receptors on these cells, leading to increased tyrosine phosphorylation and transformation (22). Thus is seems that the physiological tyrosine phoshorylation signal for proliferation in response to growth factors has been co-opted by transforming viruses in two ways: by producing truncated receptors which have constitutive unregulated kinase activity, and by producing a ligand which can stimulate a cell's endogenous receptors.

In addition to these oncogenes related to growth factor receptors, a number of cell-derived viral tyrosine kinases seem unrelated to receptors. These include the first discovered tyrosine kinase, the *src* protein pp60, as well as the products of the *abl*, *fes/fps*, *fgr*, *ros*, and *yes*

genes. Although the function of these proteins in normal cells is unknown, they may be related to changes that occur in the development and differentiation of a cell. For example, it is known that fertilization in sea urchin eggs is accompanied by a 5- to 10-fold increase in tyrosine phosphorylation (23) and that the microinjection of pp60 into Xenopus laevis oocytes accelerates maturation (23a). The steps in Mullerian duct regression may also be controlled through changes in tyrosine phosphorylation (24). It now seems that cells possess two levels of tyrosine kinases: one related to growth factor receptors which gives a cell the ability to respond to immediate changes in proliferation signals, and another unrelated to receptors, which governs the overall growth and differentiation program of a cell. In either case, tyrosine phosphorylation, as opposed to serine or threonine phosphorylation, seems directly related to proliferation.

Relationship of tyrosine phosphorylation and normal and neoplastic cell growth. An important question is how the tyrosine kinases of tumor viruses -- which were appropriated from host cells -- differ from their normal counterparts. The two possibilities are quantitative changes (overexpression of a normal protein) or qualitative changes (normal expression of an altered gene product), or some combination of the two. This question has been explored in greatest detail with the src protein pp60, and

the evidence suggests that qualitative changes are the critical ones. Parker et al. (62) demonstrated that merely increasing the expression of the normal cellular src gene could not lead to tranformation. It was subsequently shown (63) that the carboxyl terminus of the viral protein differs from the cellular product in that it lacks a tyrosyl residue which is autophosphorylated. This suggested that that particular autophosphorylation might be a negative feedback regulator, and that its absence in the viral protein could allow unrestrained kinase activity. It is also known that the middle T antigen of the polyoma virus causes transformation by increasing the kinase activity of the cellular pp60; this activation was also shown to be secondary to a qualitative change in the protein rather than an increase in overall production (64). Thus, at least for the src product, it appears that qualitative changes rather than increased production is essential for activation. Whether such changes lead to altered substrate specificity, kinetics, cell-cycle specificity, or sub-cellular localization is unknown. The latter possibility is attractive as it has been shown that amino-terminal myristylation of pp60 is necessary for its membrane assocation, and mutants lacking this modification no longer have transforming ability (65).

Tyrosine kinases in human tumors. Given the importance of tyrosine phosphorylation in retroviral-mediated

tumorigenesis, the question arises as to the significance of this modification in naturally occurring human neoplasms. Jacobs and Rubsamen (66) screened thirty carcinomas and sarcomas and compared the tyrosine kinase activity of pp60 from biopsy specimens to that of appropriate normal tissue. They found elevated activity (4- to 20-fold) in one-third of the tumors they examined. Fischer and colleagues screened 24 leukemias, and found elevate tyrosine kinase activity in the membrane fraction of only one (67). Others have examined a variety of leukemias and found either no difference, or lower tyrosine kinase activity in the tumor cells as compared to their normal counterparts (68-70). Using a slightly different approach, Lin et al. (71) examined tyrosine kinase activity in the serum of patients with malignancies. They found elevated levels in two of six patients with skin cancer and 10 of 12 with malignant melanoma, but no elevation in patients with either renal or colon carcinomas. Although these studies suggest a correlation between elevated tyrosine kinase activity and certain malignancies, the association is far from uniform.

Evidence from chromosomal studies, however, indicates that alterations in the genes for tyrosine kinases may be of great importance. Many neoplasms are found to have chromosome rearrangements, and many human hematologic malignancies have been shown to have alterations in chromosome 20, particularly the long arm. As the cellular

src gene has also been mapped to the long arm of chromosome 20 (as well as the short arm of chromosome 1), LeBeau (72) et al. examined the fate of the *src* sequence in three patients with hematologic malignancies and deletions in this chromosomal region. Despite extensive interstitial deletions in the region of the *src* gene, this proto-oncogene was retained in all of the cases. These data, with the previous results on chromosome 20, suggest that the *src* gene may be important in hematologic malignancies.

Chromosomal analysis in another system has also lent support for the significance of tyrosine kinases in human tumors. Chronic myelogenous leukemia (CML) cells are characterized by the presence of the so-called Philadelphia chromosome, a translocation involving chromosome 9. The cellular *abl* oncogene, which codes for a tyrosine kinase is located on chromosome 9, and is involved in the translocation. It has further been shown that CML cells contain a unique *abl*-related mRNA, and that during the blast crisis stage of the disease, these cells contain up to eight times higher levels of the transcript than during the chronic phase (73). This evidence again points to the potential importance of another tyrosine kinase in a discrete group of neoplasms.

Taken together, then, there is evidence for both qualitative and quantitative changes being important in the activation of tyrosine kinases in human tumors. While it is

clear that no uniform change in enzymatic activity is present throughout various tumor types, a wide range of malignancies do show abnormalities in tyrosine kinase activity. Furthermore, changes in substrate specificity, sub-cellular localization, or cell-cycle expression, which would not be detected in these studies, may be of prime importance. Thus, in addition to the alterations in phosphotyrosine regulation already demonstrated, other, more subtle defects may exist as well.

Phosphotyrosine phosphatases. One other aspect of tyrosine phosphorylation which has received somewhat less attention concerns phosphotyrosine phosphatases. As mentioned above, temperature-sensitive mutants of the src gene were of great importance in dissecting the function of these viral oncogenes. The fact that switching to the non-permissive temperature led to such a rapid reversal of the transformed phenotype (74) suggested that the critical modification, now know to be tyrosine phosphorylation, must be rapidly reversible. Similarly, mitotic stimulation by hormones such as EGF, PDGF, and insulin are quickly terminated upon removal of the hormone. This evidence suggests that phosphotyrosine phosphatases must be active in these cells; such a finding is not surprising given the critical nature of this modification.

In 1981, two groups discoverd protein phosphotyrosine phosphatase activity, one in tumor cells (75) and one in

normal mammalian tissue (76). These activities were distinguished from other known phosphoserine phosphatases (such as phosphorylase phosphatase) by their inhibition by Zn^{2+} and insensitivity to F^-. An interesting extension of this work was the finding that several of the so-called acid and alkaline phosphatases possessed phosphotyrosyl phosphatase activity (77, 79). Acid and alkaline phosphatase activity is generally measured by the hydrolysis of p-nitrophenyl phosphate (PNPP), which is structurally related to phosphotyrosine. Swarup et al. (77) compared the substrate specificities of three alkaline phosphatases (two eukaryotic one prokaryotic) and a phosphoprotein phosphatase from rabbit muscle. They found that the alkaline phosphatases were able to dephosphorylate phosphotyrosine as well as PNPP, yet they were inactive against phosphoserine-containing substrates; furthermore, purification procedures demonstrated that the phosphotyrosine and PNPP phosphatase activities resided on the same protein and that the phosphotyrosine phosphatase was active at a physiological pH. Conversely, the rabbit phosphoprotein phosphatase lacked activity against phosphotyrosine or PNPP, yet it was able to cleave phosphoserine-containing substrates.

This finding of a physiological function for the widespread alkaline phosphatases also suggested an explanation for the modulation of this activity known to occur in certain tumor cells. Benham et al. (78), for

example, showed that when HeLa cells were grown in tissue culture then passaged in nude mice, the level of one alkaline phosphatase isozyme decreased markedly; upon transfer back to an *in vitro* system the enzyme level increased. Such modulation may reflect a change in the regulation of intracellular phosphotyrosine levels; growth in the animal would select for more malignant cells which would presumably have greater levels of phosphorylated tyrosine, and thus lower levels of a phosphotyrosine phosphatase.

As phosphotyrosine phosphatases would seem to function in a reciprocal complementary fashion with tyrosine kinases, inhibition of the phosphatase activity would be expected to have the same effect as increasing the kinase activity. Employing rat kidney cells, Klarlund (80) showed that vanadate, a potent phosphotyrosine phosphatase inhibitor, increased intracellular phosphotyrosine levels by 40-fold. This was accompanied by neoplastic transformation as assessed by morphological, biochemical, and physiological changes; all of these alterations were reversed upon removal of the vanadate. Others have shown that alkaline phosphatase inhibitors stimulate bone cell proliferation, though the interaction is complex (81). Vanadate has also been shown to establish euglycemia in diabetic mice in the presence of low insulin levels, perhaps by preventing the dephosphorylation of tyrosine residues phosphorylated by the

insulin receptor kinase (82). Other evidence, however, suggests that at least some of the effects of vanadate are not mediated via phosphatase inhibition but rather through a direct stimulation of tyrosine phosphorylation (83). Tracey and Gresser (84) have shown that the vanadate ion can directly esterify the hydroxyl group of a phenyl ring, and could directly form a tyrosyl-vanadate link on cellular tyrosine kinases. Thus vanadate could activate these cellular enzymes in a manner analogous to autophosphorylation, as discussed above.

One final comment on phosphotyrosine phosphatases concerns their potential role in the development of human tumors. Certain forms of cancer, including retinoblastoma and Wilms' tumor, are known to have a strong hereditary component. Patients with these diseases are believed to inherit one abnormal allele at the locus of a so-called recessive oncogene. If they acquire a second abnormal allele at this locus, they develop a tumor (85, 86). Generally this is the result of the loss of a functional gene product. An excellent candidate for such a gene product would be a phosphotyrosine phosphatase. The loss of such an enzyme in a cell with normal tyrosine kinase activity might lead to the unbalanced accumulation of phosphotyrosine residues with resultant transformation. This would represent transformation by a recessive mechanism, as opposed to the better delineated dominant

oncogenes which would transform (as an example) by the overexpression of a tyrosine kinase. The functional end product of these two mechanisms -- a buildup of cellular protein phosphotyrosine and neoplastic transformation -- would be the same.

Relationship of proliferation and differentiation. Although the study of the proliferative process has aided greatly our understanding of the development of neoplasms, tumor formation in the animal may also be viewed as a defect in the complementary process of differentiation. All living tissue in the organism is in a homeostatic balance between proliferating, immortal, multipotential stem cells and non-dividing, specialized cells with a finite lifespan -- so-called terminally differentiated cells. This arrangement allows the animal to constantly produce functional end-stage cells, and affords it the ability to rapidly respond to a diverse array of external signals. The best studied example of such a system is the mammalian hematopoietic system. All of the formed blood elements, namely the erythrocytes, leukocytes (granulocytes, lymphocytes, and monocytes), and thrombocytes, arise from a single stem cell. That this is the case has been conclusively shown in experiments in which an animal was lethally irradiated destroying all of its hematopoietic cells. All of the blood cells were reconstituted by the transfer of a single stem cell, and the recipient hematopoietic cells all bore the marker of the

donor cell (87). The regulation of the proliferation and differentiation of these cells must be exquisite, such that an animal can respond to decreased oxygen by erythrocyte production, bacterial infection by neutrophil production, viral infection by lymphocyte production, parasitic infection by eosinophil production, and return rapidly to the baseline state upon removal of the stimulus. Both proliferation and differentiation are critical for this control. The loss of the ability of a single immature cell to proliferate would not have deleterious effects on the host, as the large number of other stem cells could easily replace the decrement. The loss of the ability of a single immature cell to differentiate, however, could lead to a catastrophic result. The uncontrolled proliferation of such a clone would result in the crowding out of normal cells, with extremely deleterious effects. Despite very rigid control mechanisms, such a process occurs in the development of a leukemia. Although such tumors may be viewed as arising from an abnormality in the proliferative response, they may also be seen as developing from a loss in the appropriate response to signals to differentiate, in which the proliferative capacity is shut down in a programmed fashion.

Evidence exists, however, to show that such a block of differentiation is not necessarily irreversible. As early as 1974, Brinster (88) showed that teratocarcinoma cells

injected into blastocysts could participate in normal development, and form normal tissues in the resultant chimeric animal with no evidence of malignant cells. Several years later is was shown that myeloid leukemic cells could be injected into 10 day-old mouse fetuses and produce normal granulocytes in the mature mice (89). Interestingly, when such cells were injected into 11 day-old fetuses, they did not participate in normal granulopoiesis, and some of the recipients developed tumors. Lotem and Sachs (90) have demonstrated that the injection of a differentiation-inducing protein in mice could inhibit the development of leukemia by several cell lines.

The HL-60 promyelocytic leukemia model. Given this background, it is clear that the study of differentiation is not only important for understanding normal cellular regulation, but also for elucidating the development of neoplasms, and perhaps for their therapy as well. As such, the development of an appropriate model system for studying hematopoietic differentiation *in vitro* was of prime importance. Of the several developed over the past 10 years one of the most widely used is the HL-60 promyelocytic leukemia. This cell line was established from the peripheral blood of a patient with acute promyelocytic leukemia (19). These cells have the valuable property that they can mature along two divergent pathways. Agents such as dimethyl sulfoxide (DMSO), butyric acid, dimethyl

formamide (92), retinoic acid (93), and anthracyclines (94) induce differentiation along the granulocytic lineage, predominantly into neutrophils. Monocytic differentiation can be induced by compounds that include the phorbol ester 12-O-tetradecanoyl 13-acetate (TPA) (95), 1,25-dihydroxy-vitamin D_3 (96), and cytosine arabinoside (97). Maturation into eosinophils, a granulocyte subset, has been shown to occur upon exposure to alkaline medium (pH 7.8) (98). This capability to differentiate along these divergent lineages allows the study not only of the progession from immature to mature cell, but also the biochemical mechanisms which distinguish the "choice" to progress down one pathway or the other. That these cells can, in fact, mature indicates that whatever the initial leukemic lesion was, it can, to some extent, be reversed.

The phenotypic changes which accompany differentiation have been well described. Granulocytic maturation of HL-60 cells leads to changes in morphology (decreased cell size, nuclear condensation and segmentation), changes in the plasma membrane (loss of transferrin receptor and other glycoproteins, altered fluidity and ion fluxes), changes in metabolism (induction of hexose monophophate shunt and superoxide production, increased phagocytosis and polyamine accumulation), as well as a number of other modifications (99). Monocytic differentiation is accompanied by a similar array of changes, including induction of non-specific

esterase activity, increased cell-cell and cell-substratum adherence, and development of tumoricidal capabilities (99). For all of the understanding of the changes that occur with differentiation, rather little is known about the mechanism(s) by which the diverse agents noted above induce this process. Such knowledge would be essential in formulating chemotherapeutic regimens specifically aimed at the induction of terminal maturation.

The fact that the agents which induce differentiation are structurally diverse and act at concentrations six orders of magnitude apart immediately suggests that these drugs act at a variety of sites. Among those that induce granulocytic maturation, the plasma membrane appears to be one target. DMSO, for example, is lipophilic, and alters ion fluxes in HL-60 cells (100). In erythroid systems, agents which interact with the plasma membrane such as procaine (a local ansthetic) and amiloride (a Na^+-H^+ antiporter inhibitor) block differentiation (101, 102). In addition, agents which block DNA replication such as cytosine arabinoside (103), 6-thioguanine (104), and aphidocolin (103) induce HL-60 maturation. 5-Azacytidine, which blocks DNA methylation, is also active (105). Finally, agents such as the anthracycline compounds -- which are known to affect DNA metabolism, the plasma membrane, and glycoprotein biosynthesis -- cause differentiation as well (106). Attempts to determine a specific portion of the cell cycle

during which these drugs exert their effect have also shown a spectrum of results. Retinoic acid appears to induce commitment to differentiate principally during late S phase (107), sodium butyrate at the G_2-M boundary or early G_1, (108), and DMSO apparently acts independent of the cell cycle (109). The data for monocytic differentiation, which is expressed in half the time that it takes for granulocytic maturation to occur, are equally complex. Dihydroxyvitamin D_3 may be acting via a specific intracellular receptor (110); TPA stimulates protein kinase C, though this alone is not sufficient to induce differentiation, and one or more undefined actions are also necessary (111).

For all of this diversity, it seems likely that these agents activate pathways which ultimately converge on a common differentiation program at the DNA level. This program involves the shutdown of the expression of genes necessary for proliferation, and the activation of those needed for the mature phenotype (either granulocytic or monocytic).

Although maturing HL-60 cells are not functionally identical to normal mature granulocytes (112) and monocytes (110) they are quite similar phenotypically. In one critical respect they are identical and that is that the tumor cells become terminally differentiated, with a short lifespan and no proliferative capacity. The elucidation of this aspect is essential for understanding the maturation

process as well as for exploiting differentiation for therapeutic uses. Changes in the expression of oncogenes have received much attention in this regard. HL-60 cells are known to have two activated oncogenes: myc, which is amplified and overexpressed (113,114), and N-ras which contains a point mutation, and can transform NIH-3T3 fibroblasts (115). The myc oncogene codes for a nuclear binding protein while the ras gene product is a 21,000 MW membrane bound GTP binding protein (116). Granulocytic maturation is accompanied by a decrease in myc expression (114), but no change in that of N-ras (117). Monocytic differentiation leads to a similar fall in myc expression with no change in N-ras (117) but, in addition, there is a pronounced increase in the expression of the fos oncogene which is undetectable in the immature cells (118). This is the transforming gene of a murine osteosarcoma virus (114), although high levels of this product have been found in mature monocytoid cells (118). Thus, although something is known about the mechanisms of action of some of the inducers and about the changes in oncogene expression which accompanies maturation, little is known about the central biochemical changes which underlie differentiation.

Relationship of tyrosine phosphorylation and differentiation. As tyrosine phophorylation appears intimately linked to the signal for proliferation, and as the shutdown of proliferation is an essential aspect of

differentiation, the regulation of tyrosine phosphorylation during differentiation would seem to be an important area for study. The earliest work in this area focused on erythroid models of differentiation. Graf and coworkers infected erythroblasts with a mutant avian erythroblastosis virus (AEV) (119). This virus contained a temperature sensitive mutation in its oncogene (now known to be a tyrosine kinase), and had a decreased tumorigenic potential at elevated temperature. At 35°C the infected erythroblasts were transformed, and lacked any evidence of a mature phenotype. Raising the temperature to the non-permissive temperative (41°C) inactivated the kinase and allowed expression of a differentiation marker, namely hemoglobin synthesis. Thus an active viral tyrosine kinase kept the cells in an immature state, and inactivation was followed by maturation. Kahn et al. (120) extended this work to show that viruses carrying other kinases (*src* and *fps*) could transform erythroid cells. They found, however, that some spontaneous differentiation did occur; nevertheless, infection with temperature sensitive mutants of these viruses followed by incubation at the non-permissive temperature allowed widespread terminal erythrocytic differentiation of the population.

Hines (121) examined promoncytic cells infected with the Abelson murine leukemia virus (121), another retrovirus bearing a tyrosine kinase. These cells were able to cause

tumors in syngeneic mice, yet they also possessed some ability to differentiate spontaneously. This maturation could be augmented further by the addition of physiological differentiation factors. In these cases, then, the active tyrosine kinase caused transformation, yet differentiation, both spontaneous and induced, could occur as well. Thus, in contrast to the erythroblastic model of Graf et al. (119), the block in differentiation evident in these latter models was not complete.

In order to examine tyrosine kinase-induced changes in hematopoietic differentiation in a more physiological setting, workers in the laboratory of Dexter (122) developed long term bone marrow cultures, then infected them with a Rous sarcoma virus derivative. The authors found a shift in the distribution of cells in the granulocytic pathway, with a decrease in mature cells and an increase in immature and stem cells. Although the stem cells had a greater self-renewal capacity than uninfected cells, when injected into lethally irradiated animals they were able to reconstitute the hematopoietic system without forming tumors in the recipients. Thus, after these cells received a demonstrably active tyrosine kinase, they showed a shift towards self-renewal at the expense of differentiation, yet the block in maturation was not complete.

Relatively little work has been done in examining the effects of tyrosine kinases on non-hematopoietic

differentiation. Falcone et al. (123) infected myoblasts with a temperature sensitive mutant of Rous sarcoma virus. Analagous to findings in erythroblasts, at the permissive temperature the cells remain transformed myoblasts, while at the elevated temperature they differentiate into multinucleated myotubes, expressing muscle-specific myosin, desmin, and acetylcholine receptors.

The central nervous system is populated by cells with little to no growth potential, yet paradoxically this most highly differentiated tissue possesses considerable tyrosine kinase activity (124). The functional significance of this is unclear. To explore further the relationship of tyrosine phosphorylation and neuronal differentiation, Alema et al. (125) studied a rat pheochromocytoma cell line. When these cells are treated with nerve growth factor (NGF), they respond by shifting from an immature chromaffin cell phenotype to that of a mature neurite-bearing sympathetic neuron. Infection of these cells with a retrovirus bearing the *src* kinase, leads to a similar differentiation. It is not known whether NGF functions by activating a receptor tyrosine kinase.

Thus, it appears that in hematopoietic systems as well as in non-hematopoietic models other than the nervous system, increased tyrosine kinase activity is associated with at least a partial blockage of differentiation; decreased activity allows maturation to occur. These

studies have all examined the effects of exogenously added kinases; however, the question of endogenous control of tyrosine phosphorylation during differentiation has not been addressed.

The HL-60 promyelocytic leukemia cell line represents an excellent model system for studying differentiation, with its ability to mature along two divergent pathways, and its well defined markers. As such, it presents a good opportunity for examining the endogenous control of tyrosine phosphorylation during differentiation. This knowledge may shed light on the control of normal hematopoietic growth and maturation, the development of hematologic neoplasms, and perhaps on potential avenues of therapy for such tumors. Given this background, the current study was undertaken to examine the regulation of tyrosine phosphorylation during the differentiation of HL-60 human promyelocytic leukemia cells.

MATERIALS AND METHODS

Materials. Radioactive isotopes were purchased from Amersham Corp., Arlington Heights, IL. Tissue culture media and serum was obtained from Grand Island Biologicals Co., Grand Island, NY. All other reagents were purchased from Sigma Chemical Co., St. Louis, MO., unless otherwise noted.

Culture conditions. HL-60 promyelocytic leukemia cells were a gift from Dr. R. C. Gallo, National Cancer Institute. Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated (56 ^{o}C for 30 min) fetal bovine serum (FBS) at 37 ^{o}C in a humidified 95% air/5% CO_2 atmosphere. Cells were seeded at a level of 2 x 10^5 cells/ml, and were allowed to attain a maximum density of 1.5 x 10^6 cells/ml before being passed into fresh medium. All studies employed cells that were between passages 32 and 60.

DMSO-resistant sublines of HL-60 (HL-60/DMSO Y1 and HL-60/DMSO Y2) were derived without mutagenesis by repeated passage through growth medium containing 1.2% (v/v) DMSO, and were continuously passaged in such medium.

An HL-60 mutant subline lacking hypoxanthine-guanine phosphoribosyl tranferase (HGPRT), HL-60/HGPRT$^-$, was a gift from Dr. Kimiko Ishiguro, Yale University School of Medicine, New Haven, CT., and was passaged in HL-60 growth medium.

A431 cells were obtained from the American Type Culture

Collection (Rockville, MD). These cells were seeded at 0.5 to 1 x 10^6 cells/100 cm^2 and were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS in a humidified 90% air/10% incubator at 37°C. Cells were routinely removed from the flask by exposure to 0.05% trypsin and subcultured before reaching confluence.

WEHI-3B cells were a gift from Dr. Malcom Moore, Sloan-Kettering Institute, New York, NY. They were grown in McCoy's 5A medium supplemented with 15% (v/v) FBS and grown in a humidified 95% air/5% CO_2 incubator at 37°C. Cells were seeded at 1 x 10^5 cells/ml and passaged before reaching a density of 1.5 x 10^6 cells/ml.

Induction of differentiation. Granulocytic differentiation was induced in HL-60 cells by treatment with DMSO (1.2%, v/v), retinoic acid (RA) (10^{-6} M), aclacinomycin A (50 nM), and marcellomycin (40 nM). HL-60/DMSO Y1 and Y2 were induced to differentiate into granulocytes by RA (10^{-6} M); HL-60/HGPRT$^-$ cells were treated with 6-thioguanine (6-TG) (300 nM) for granulocytic maturation. Such differentiation was induced in WEHI-3B cells by aclacinomycin A (50 nM), Adriamycin (20 nM), and marcellomycin (30 nM).

Monocytic differentiation was induced in HL-60 cells by treatment with TPA (10 nM). 1-Oleoyl-2-acetyl-glycerol (OAG,40 mglml) was used as a negative control.

Granulocytic differentiation was measured by the ability of cells to reduce nitroblue tetrazolium (126).

Monocytic differentiation was determined by the percentage of cells containing non-specific esterase activity (127).

Cell fractionation. 4 to 5 x 10^7 cells were collected, washed twice in phosphate-buffered saline (PBS) (140 nM NaCl; 1 2,7 mM KCl; 6.7 mM $Na_2HPO_4 \cdot 7H_2O$; 1.5mM KH_2PO_4), resuspended at a level of 10^7 cells/ml in buffer A (5 mM Hepes (pH 7.4), 1 mM $MgCl_2$, and 1 mM EDTA) and disrupted with a Branson sonicator (Danbury, CT) using two 10-second bursts at a setting of 20. Nuclei and unbroken cells were removed by low speed centrifugation (1000 x g for 10 min) and the supernatant was centrifuged at 30,000 x g for 30 min. The resulting supernatant was designated the soluble fraction. The pellet was resuspended in 0.3 ml of buffer B (25 mM Hepes (pH 7.4), 5 mM 2-mercaptoethanol, and 0.1% (v/v) Nonidet P-40), shaken vigorously, and centrifuged for 5 minutes at 12,000 x g in a microcentrifuge. The resulting supernatant was designated the particulate fraction. Protein was determined by the method of Bradford (128) using bovine serum albumin as a standard.

Plasma membrane isolation. The cationic silica method of Chaney and Jacobson (129) was employed to isolate plasma membranes with only their cytoplasmic face available for molecular interactions. Three to 5 x 10^8 cells were washed twice in ice-cold 70 mM Nacl, 20 mM 2-(N-morpholino) ethane sulfonic acid (MES), pH 6.5 (wash buffer). Four milliliters of colloidal silica (gift of Dr. B. S. Jacobson, University

of Massachusetts, Amherst, MA) diluted to 6-8% (w/v) silica with attachment buffer (140 mM sorbitol, 20 mM MES, pH 6.5). The cells, in a volume of 4 ml of attachment buffer were then slowly added to the silica with gentle mixing. Immediately after mixing, the suspension was diluted to 40 ml with wash buffer. The silica coated cells were recovered by centrifugation (650 x g for 2.5 min), and resuspended in 5.0 ml wash buffer. The cells were then slowly added to 5.0 ml of a 2 mg/ml solution of polyacrylic acid (MW 90,000; Aldrich Chemical Co., Milwaukee, WI) in wash buffer (pH 6.5). After mixing, the suspension was again diluted to 40 ml in wash buffer and centrifuged as above. This procedure was repeated twice, forming a pellicle three layers thick. The cells were lysed by three cycles of rapid freezing and thawing, and the membrane fraction recovered by centrifugation (800 x g for 5 min).

Cellular membrane separation. Plasma membranes were separated from internal cellular membranes by the density isolation method of Hertel et al. (130). Cells were washed twice in PBS, then incubated with 0.25 mg/ml concanavalin A in PBS for 20 minutes at 4°C. Cells were lysed by hypotonic shock in 1 mM Tris, 2 mM EDTA, pH 7.4 for 20 minutes at 4°C. Lysates were layered on top of a step gradient consisting of 2 ml 60%, 35 ml 38%, and 3.5 ml 15% sucrose (w/v in 20 mM Tris, pH 7.4) and centrifuged for 30 minutes at 35,000 rpm. The interfaces were collected, diluted in PBS, recovered by

centrifugation, then processed as described above ("cell fractionation").

Leukocyte separation. Human blood was collected by venipuncture into glass tubes contining EDTA as an anticoagulant. The blood was mixed with an equal volume of PBS then layered on 3 ml of LSM (Litton Bionetics, Kensington, MD) and centrifuged for 40 minutes at 400 x g at room temperature. The interface contained the mononuclear cells which were further separated by adhesion to plastic (131). The cells were washed in PBS, then resuspended in McCoys 5A medium supplemented with 15% FBS, penicillin (50 u/ml), and gentamycin (50 ug/ml), and incubated on plastic plates at 10^5 cells/cm^2 at 37 °C for 90 minutes. Non-adherent cells were removed and another cycle repeated. Adherent cells were washed with PBS, then removed by the gentle application of a rubber policeman. Cells were then washed and harvested; Wright-Giemsa staining revealed that > 95% of the adherent cells had monocytoid morphology, and > 90% of the non-adherent cells resembled lymphocytes.

The pellet from the LSM separation contained neutrophils and erythrocytes. Red blood cells were removed by a combination of dextran sedimentation and hypotonic lysis. The pellet was resuspended in 1 ml of homologous plasma to which 0.4 ml of 4.5% dextran (w/v; MW 200,000) in PBS was added. The tube was inverted five times, then allowed to stand at 4 °C for 30 minutes (132). The neutrophil

enriched supernatant was recovered and the cells were washed in PBS. Remaining erythrocytes were then removed by hypotonic lysis (133). Cells were suspended in 2 ml of cold PBS, to which 6 ml of cold distilled water was rapidly added. The tube was mixed vigorously for 30 seconds, and then isotonicity was restored by adding 2 ml of 3.5% NaCl (w/v). Cells were recovered by centrifugation and washed. Hypotonic lysis was repeated up to three times until there was less than one erythrocyte per 200 leukocytes by microscopic examination. Wright-Giemsa staining revealed that greater than 98% of the leukocytes were segmented neutrophils. All cells were then processed as described above.

Phosphoaminoacid analysis. Protein and cellular phosphoaminoacids were quantitated by the method of Cooper et al. (47). Proteins and peptides labeled with ^{32}P in kinase assays (see below) were precipitated in ice-cold 10% (w/v) trichloroacetic acid (TCA), resuspended in 6N HCl, and hydrolysed for 90 minutes at 110°C. HCl was removed by vacuum centrifugation, and the pellet was resuspended in 5 ul of thin layer electrophoresis (TLE) buffer (pyridine: glacial acetic acid: water, 10:10:1890, pH 3.5) containing 0.5 ug each of unlabeled phosphoserine (P-ser), phosphothreonine (P-thr), and phosphotyrosine (P-tyr). Samples were applied to cellulose coated (250 um) glass plates and separated by the application of 800 V for 45

minutes at 4°C. The plates were then dried, the standards visualized with ninhydrin, and the spots scraped, with radioactivity determined by liquid scintiliation spectroscopy.

To assess whole cell phosphoaminoacid distribution, cells were labeled with [^{32}P] orthphosphate for 16 hours in RPMl 1640 medium lacking phosphate but supplemented with 4% FBS, penicillin (50 U/ml), gentamicin (50 ug/ml), and glutamine (2 mM). Cells were then washed twice in PBS, and lysed in lysis buffer (1% sodium dodecyl sulfate (w/v, SDS), 5 mM EDTA, 150 mM NaCl, 100 kallikrein inhibitory units/ml aprotinin, 1 mM phenylmethy/sulfonyl fluoride, 10 mM Tris-HCL, pH 7.4) with vigorous shaking at 4°C. Unbroken cells and debris were removed by centrifugation (20,000 x g for 20 minutes at 2°C). Nucleic acids, phospholipids, and low molecular weight compounds were subsequently removed by phenol extraction and precipitation.

The supernatant from the clearing centrifugation was mixed with 0.4 ml NTE (0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, 1 mM EDTA) and 0.4 ml of buffer saturated phenol at room temperature. The solutions were mixed vigorously and centrifuged at 12,000 x g for 1 minute. The phenol layer was extracted once again with 0.8 ml of NTE, and then mixed with 13 ml of water and 2 ml of 100% (w/v) TCA, and allowed to stand at 2°C for 1 hour. The precipitate was recovered by centrifugation (10,000 x g for 10 minutes at 2°C), extracted

with 5 ml of $CHCl_3$: methanol, 2:1 (v/v), and centrifuged as before. The pellet was then hydrolysed and subjected to electrophoresis as above, with the exception that electrophoresis at pH 3.5 was preceded by electrophoresis at pH 1.9 (formic acid: acetic acid: water, 50:156:1794, v/v) in a perpendicular direction. This additional step was needed to separate P-tyr from uridine monophosphate. Spots were then isolated and quantitated as above.

Tyrosine kinase assay. Tyrosine phosphorylation of artificial substrates was measured by a modification of the method of Braun et al. (134). Ten to 20 ug of protein was added to the tyrosine kinese buffer containing 20 mM Hepes (pH 7.4), 12 mM $MnCl_2$, 10 uM $ZnCl_2$, and 0.5% (v/v) Nonidet P-40 (v/v) with or without substrate. After 3 minutes at 22°C, the reaction was initiated by the addition of [γ-^{32}P] ATP (3 Ci/mmol) to 25 uM. The reaction was terminated by the addition of 7 ul of unlabeled ATP (10mM). Fifty microliters of the mixture was then applied to a 1 cm x 1 cm square of Whatman 3MM filter paper, which was washed and counted as described by Corbin and Reimann (135), except that the TCA washes contained 10 mM sodium pyrophosphate. When angiotensin II was used as substrate, the kinase reaction was stopped by the addition of 21.4 ul acetic acid (30% v/v final); 65 ul of the mixture was then applied to 2 cm x 2 cm squares of Whatman P81 phosphocellulose ion-exchange paper. The squares were then washed three

times in 15% acetic acid (v/v), once in acetone, and then dried. In each case, radioactivity was assessed by liquid scintillation spectrometry, and net phosphorylation represented the difference between tubes with and without substrate.

Protein phosphotyrosine phosphatase assay. Protein phosphotyrosine phosphatase was measured using a modification of the method of Shriner and Brautigan (136). The substrate [Tyr (^{32}P)] glutamic acid: alanine: tyrosine, 6:3:1, (GAT), was prepared by incubating 20 to 30 ug of A431 cell particulate fraction protein with 1 to 2 mg of GAT, 10^{-7} M EGF (Collaborative Research, Lexington, MA), and 25uM Na_3VO_4 in a total volume of 1 ml of tyrosine kinase buffer. The reaction was allowed to proceed overnight at 22°C and was terminated by the addition of 111 ul of 100% (w/v) TCA. A precipitate was allowed to form at 4°C for 1 hour and the pellet was collected by centrifugation at 12,000 x g for 5 minutes. The pellet was washed three times in 10% TCA at room temperature and was solubilized in 100 ul of 1 M NaOH. Two milliliters of protein phosphotyrosine phosphatase buffer (50 mM Hepes, pH 7.0, and 25 mM 2-mercaptoethanol) were added and the solution was diafiltrated repeatedly (10,000 MW cutoff; Amicon Corp., Danvers, MA) at 5,000 x g for 30 minutes against the buffer until the ^{32}P in the filtrate was 1% of that of the retentate. The retentate was then collected and designated

the protein phosphotyrosine phosphatase substrate.

Protein phosphotyrosine phosphatase activity was measured by the release of [^{32}P] orthophosphate from [Tyr(^{32}P)] GAT in a 50 ul reaction mixture containing approximately 10,000 cpm of substrate and 10 to 20 ug of protein in protein phosphotyrosine phosphatase buffer containing 0.5 mg/ml of bovine serum albumin at 37°C. The reaction was initiated by the addition of the substrate and was allowed to proceed for one minute, at which time the reaction was terminated by the addition of 50 ul of 20% TCA. The tube was mixed well, chilled at 4°C for 5 minutes, and centrufuged at 12,000 x g for 5 minutes. Fifty microliters of the supernatant was mixed with 5 ml of Hydrofluor (National Diagnostics, Sommerville, NJ) and radioactivity therein was determined with a scintillation spectrometer. Specific activity was represented as the difference in the amount of radioactivity released in the presence and absence of substrate.

To ensure that the ^{32}P released was the result of phosphatase activity rather than protease activity, a molybdate-organic solvent extraction was performed (76). This technique quantitatively separates inorganic phosphate from phosphorylated amino acids and peptides. Forty microliters of the supernatant from the TCA-precipitated reaction mixture was added to 80 ul of 1.25 mM KH_2PO_4 in 0.5 M H_2SO_4, 200 ul of isobutanol: toluene, 1:1 v/v, and 40 ul

5% ammonium molybdate. After vigorous shaking, the upper organic phase containing the inorganic phosphate was removed. The radioactivity was determined in a scintillation counter and compared to that of an equal volume of the (non-extracted) TCA supernatant.

p-Nitrophenylphosphatase (PNPPase) assay. PNPPase activity was determined by a modification of the method of Shriner and Brautigan (136). Ten to 20 ug of protein were incubated in a total volume of 90 ul of protein phosphotyrosine phosphatase buffer at 37°C for 3 minutes. The reaction was initiated by the addition of 10 ul of neutralized 0.1 M p-nitrophenylphosphate. The reaction was allowed to proceed for the indicated length of time, then terminated by the addition of 0.9 ml of 1 M sodium carbonate. The absorbance at 410 nm was measured. Phosphatase activity was represented as nanomoles of p-nitrophenol formed per min per mg protein, using a millimolar extinction coefficient of 17.5 for p-nitrophenol at 410 nm and pH 11.

Protein purification. Both tyrosine kinase and phosphotyrosine phosphatase were purified by identical strategies. Saturated ammonium sulfate (4°C) was added to particulate fractions to 25%, mixed thoroughly and incubated for 1 hour at 4°C. Precipitated protein was removed by centrifugation (1000 x g for 10 minutes); saturated ammonium sulfate was then added to the supernatant to a final

concentration of 75% saturation and again incubated at 4°C for 1 hour. The precipitate was recovered by centrifugation as above. Pellets were dissolved in buffer B (25 mM Hepes, pH 7.4, 5 mM 2-mercaptoethanol, and 0.1% (v/v) Nonidet P-40) and diafiltrated 100-fold to remove any ammonium sulfate. All activity of both enzymes was found in the fraction soluble in 25% but insoluble in 75% ammonium sulfate, and it was this fraction that was used for subsequent steps.

Column chromatographic steps were performed at 4°C by a modification of the method of Sugimoto et al. (137). The active fraction from the ammonium persulfate step was loaded onto a Sephacryl S-200 (Pharmacia, Piscataway, NJ) column (2.5-cm diameter, 100 ml bed volume) equilibrated with buffer C (buffer B containing 200 mM NaCl) at a flow rate of 60 ml/hr. The pooled active fractions were diafiltrated 100-fold with buffer B, then loaded onto a DEAE-Sephacel (Pharmacia) column (4.0 ml) equilibrated with buffer B. The column was washed with buffer B, and the protein then eluted with a linear gradient of 0 to 500 mM NaCl in buffer B. The active fractions were then combined and diafiltrated 500-fold with buffer B.

Cell volume determinations. All cell counts and volume determinations were performed on a model ZBI particle counter with an integrated channelyzer (Coulter Electronics, Hialeah, FL), using 10 um-diameter beads (Coulter) as volume standards.

Cell cycle analysis and DNA quantitation. Single cell DNA measurement and population analyses were performed on a FACS IV flow cytometer (Becton-Dickinson, Mountainview, CA) using mithramycin staining (138). Cells were fixed in 95% ethanol, then stained with a solution of mithramycin in 0.3% (w/v) $MgCl_2.6H_2O$. Flow cytometry was performed with an excitation wavelength of 457 nm and emission wavelengths greater than 520 nm.

RESULTS

Granulocytic differentiation of HL-60 cells. HL-60, when treated with DMSO or RA, differentiate from promyelocytes into mature granulocytes. After 2 to 3 days of exposure to drug, these cells begin to exhibit one of the major phenotypic markers of mature granulocytes, the ability to reduce nitroblue tetrazolium (NBT) (Figure 1). NBT is a yellow soluble dye which upon exposure to O_2^- produced by activated granulocytes is reduced to insoluble blue-black formazan. This stains the cells which produce the anion, allowing quantitation of mature cells. After 6 days of exposure to RA or DMSO a maximum of about 80% differentiated cells is seen by both the NBT assay and morphological assessment. The attainment of a mature phenotype is accompanied by decreased proliferation. After 2 days in either RA or DMSO, cells begin to exhibit a decreased growth rate, from a doubling time of 28 h in untreated cells to that of 36 h in differentiating cells (Figure 2). Furthermore, these cells reach a plateau density 25 to 40% below that of untreated cells.

This terminal differentiation (i.e., the maturation of a cell to one which can no longer divide) is also evident in the decrement of cells in the active phases of the cell cycle (Figure 3A). As early as two days after the addition of DMSO, there is a sharp decline in cells in the S, G_2, and

Figure 1. Differentiation of HL-60 cells treated with DMSO and RA. Cells were exposed to 1.2% (v/v) DMSO (Δ), 10^{-6} M RA (O), or untreated (●), and the ability to reduce nitroblue tetrazolium was assessed as described in "Materials and Methods." Each point represents the mean ± standard error of the mean of three separate experiments each done in duplicate.

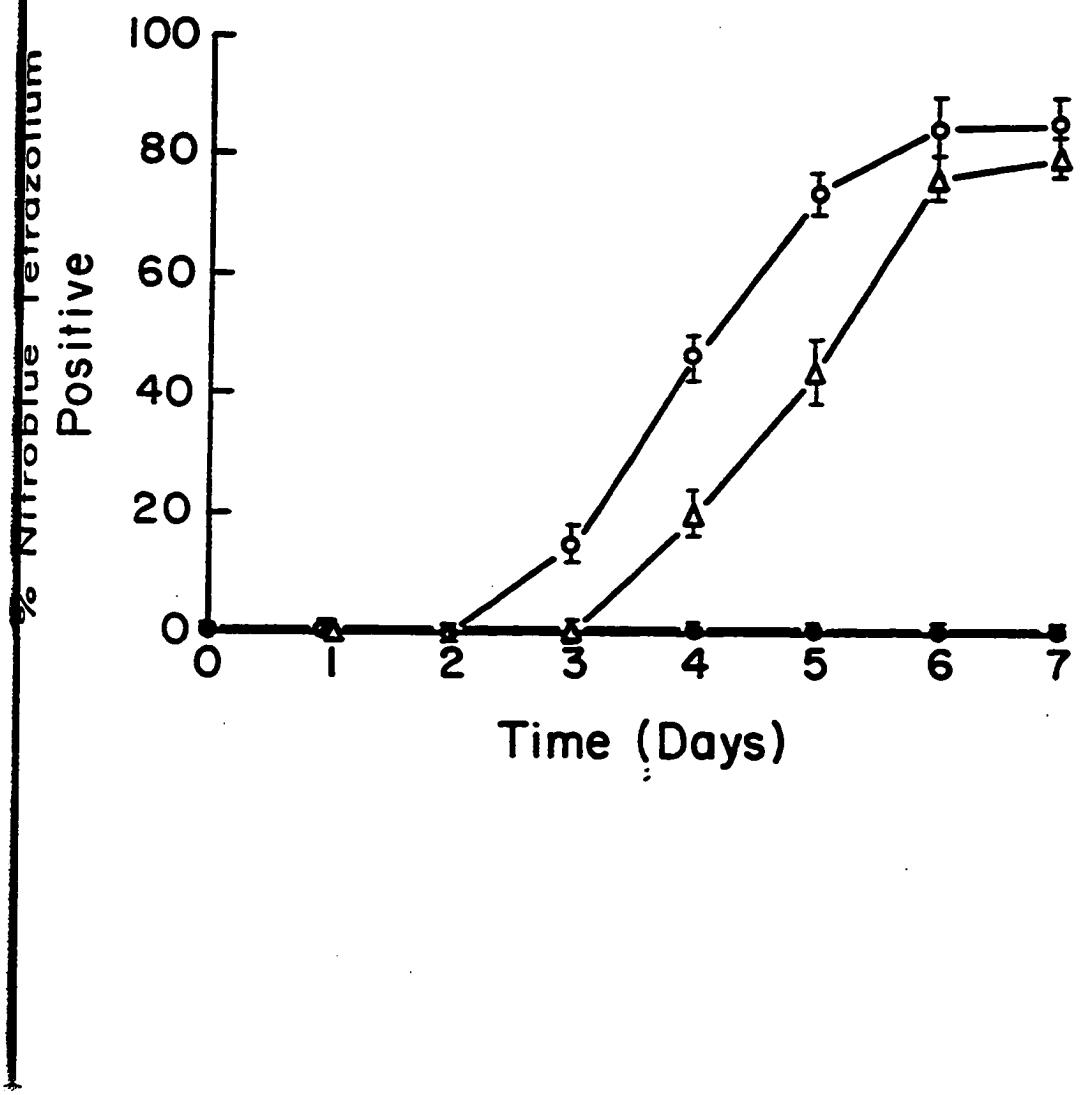


Figure 2. Growth of HL-60 cells treated with DMSO and RA. Cells were exposed to 1.2% (v/v) DMSO, (△), 10^{-6} M RA (○), or untreated (●), and cell counts were determined at each indicated time using a coulter ZBl Particle Counter. Viability was consistently greater than 90% as assessed by trypan blue exclusion. Each point represents the mean ± standard error of the mean of three separate experiments each done in duplicate.

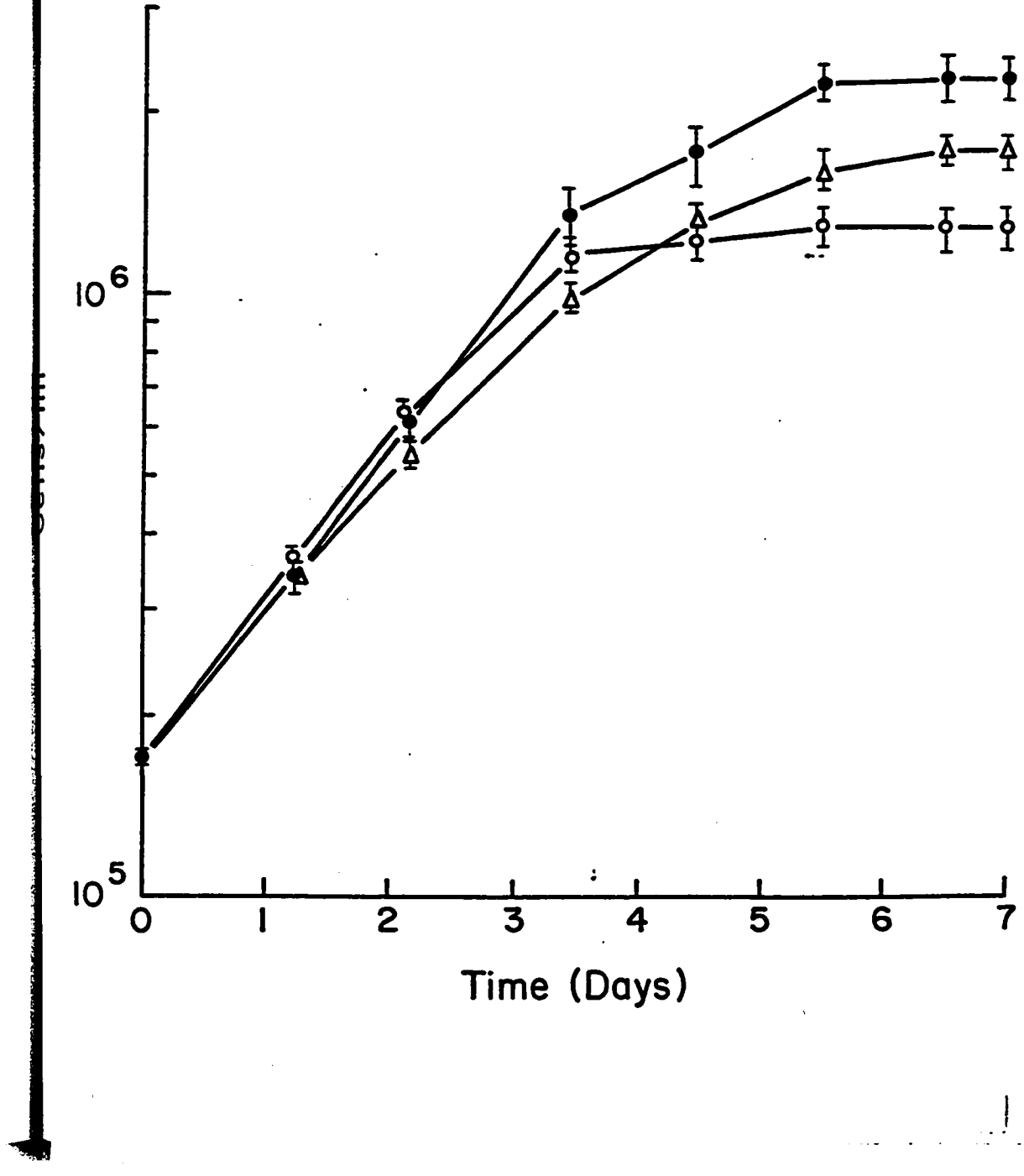
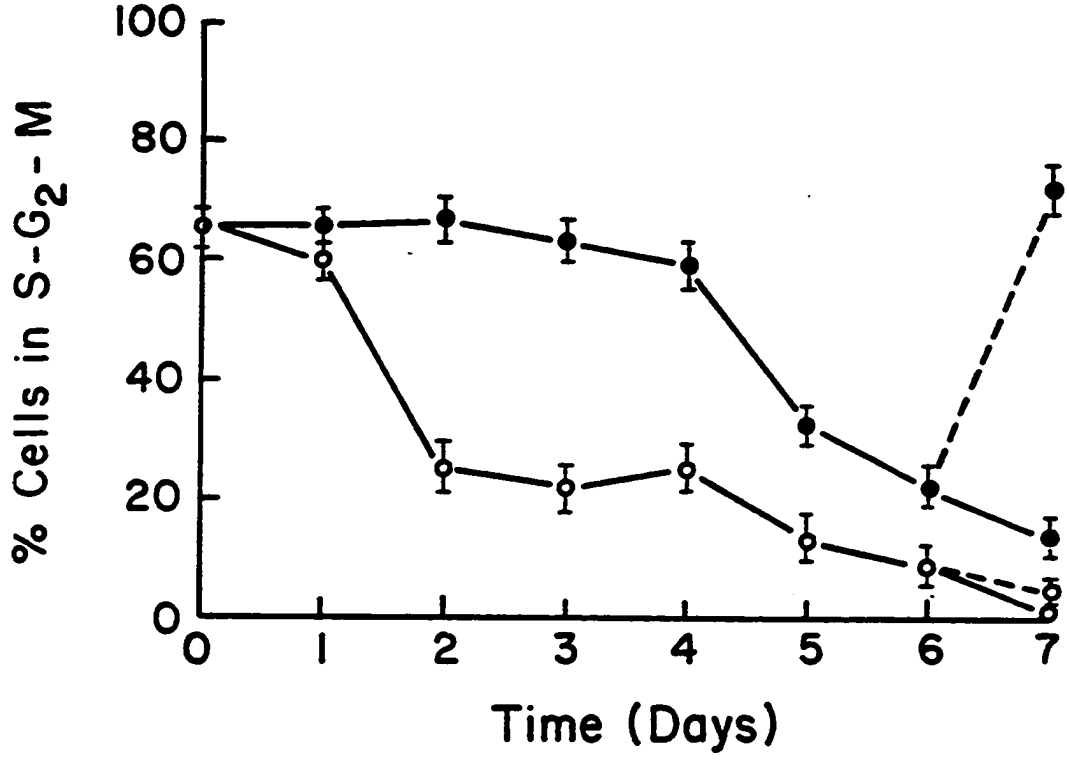


Figure 3A. Proportion of HL-60 cells in the proliferative phases of the cell cycle during DMSO-induced differentiation. Cells were treated with 1.2% (v/v) DMSO (O), and the percentage of cells in the S, G_2, and M phases of the cell cycle determined as described in "Materials and Methods." After 6 days, an aliquot of cells was placed in fresh medium, and a separate determination made one day later (---). Each point represents the mean ± standard error of the mean of two separate experiments each done in duplicate.



M phases of the cycle. Although untreated cells also eventually show a decline in such a cycling population, this effect is rapidly reversed by placing the cells into fresh medium; no such phenomenon is observed with the differentiating cells. Similarly, the average DNA content of the maturing cells falls precipitously, and cannot be reversed by medium replenishment; the eventual decline in DNA content of control cells is readily reversible (Figure 3B).

As cells progress along the myeloid pathway, they show a decrease in cell volume. This represents a combination of two factors: the condensation of the nucleus and the buildup of cells in the G_1 phase of the cell cycle. Figure 4 demonstrates the greater than 50% decrease in mean cellular volume following DMSO treatment. Untreated cells also show a fall in volume after 5 days in culture as they plateau, but this effect is readily reversible with medium replenishment, and the return of the cells to the proliferating portions of the cell cycle. In tandem with the decrease in volume, these cells also exhibit a decrease in protein content (Figure 5). As such, the ratio of cellular protein to cell volume does not vary by more than 15% with DMSO-induced differentiation. Again, fresh medium reverses the decline seen in untreated cells which reach saturation density.

Figure 3B. Mean cellular DNA content of HL-60 cells during DMSO-induced differentiation. Employing the same data as those used to generate Figure 3A, mean DNA content was determined in untreated cells (●), and cells treated with 1.2% (v/v) DMSO (○). See legend of Figure 3A for experimental detail.

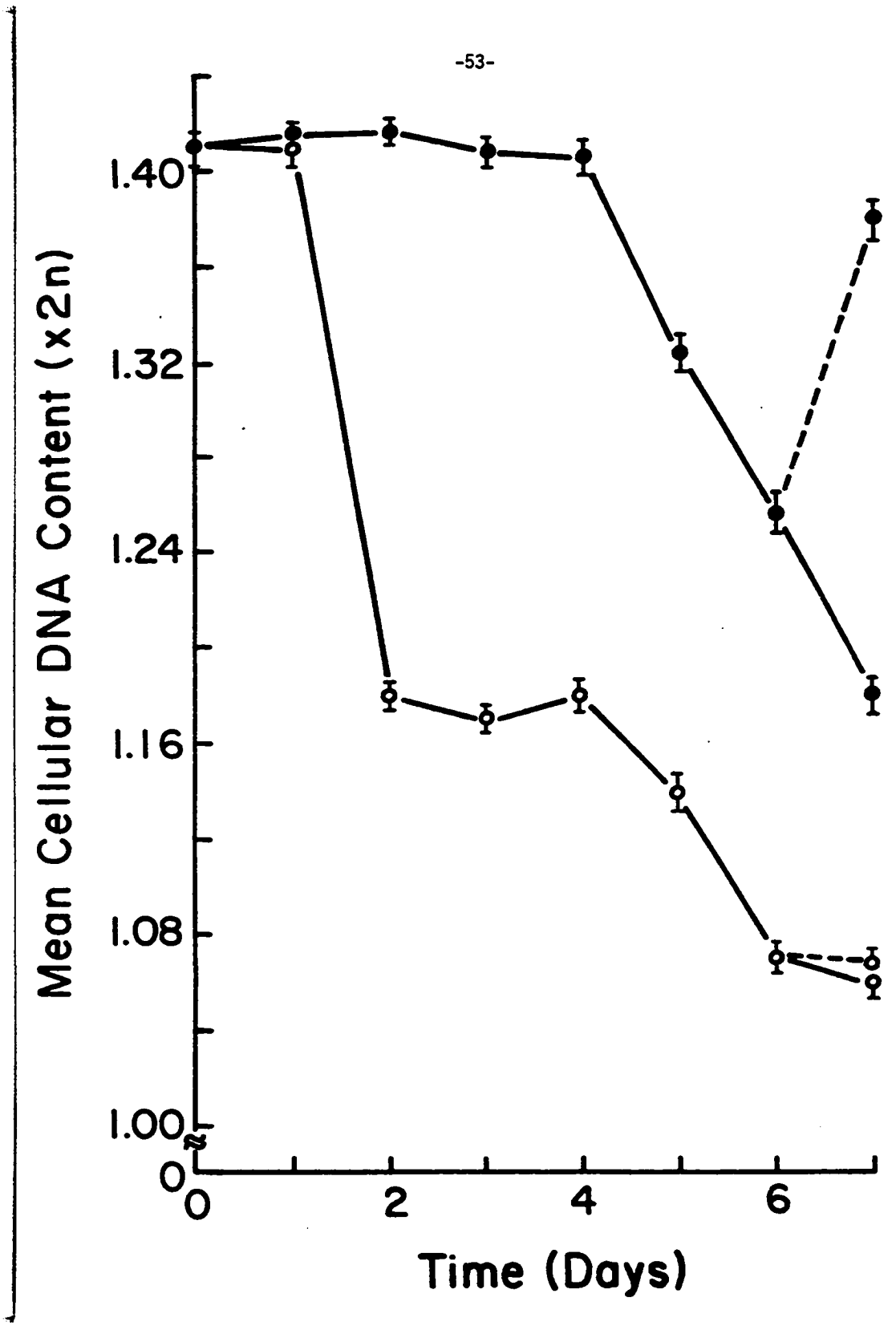


Figure 4. Cellular volume in HL-60 cells during DMSO-induced maturation. Cells were treated with 1.2% (v/v) DMSO (o) or untreated (●), and the mean cellular volume was determined as described in "Materials and Methods." After 6 days, an aliquot of cells was placed in fresh medium, and a separate determination made one day later (---). Each point represents the mean ± standard error of the mean of three separate experiments each done in duplicate.

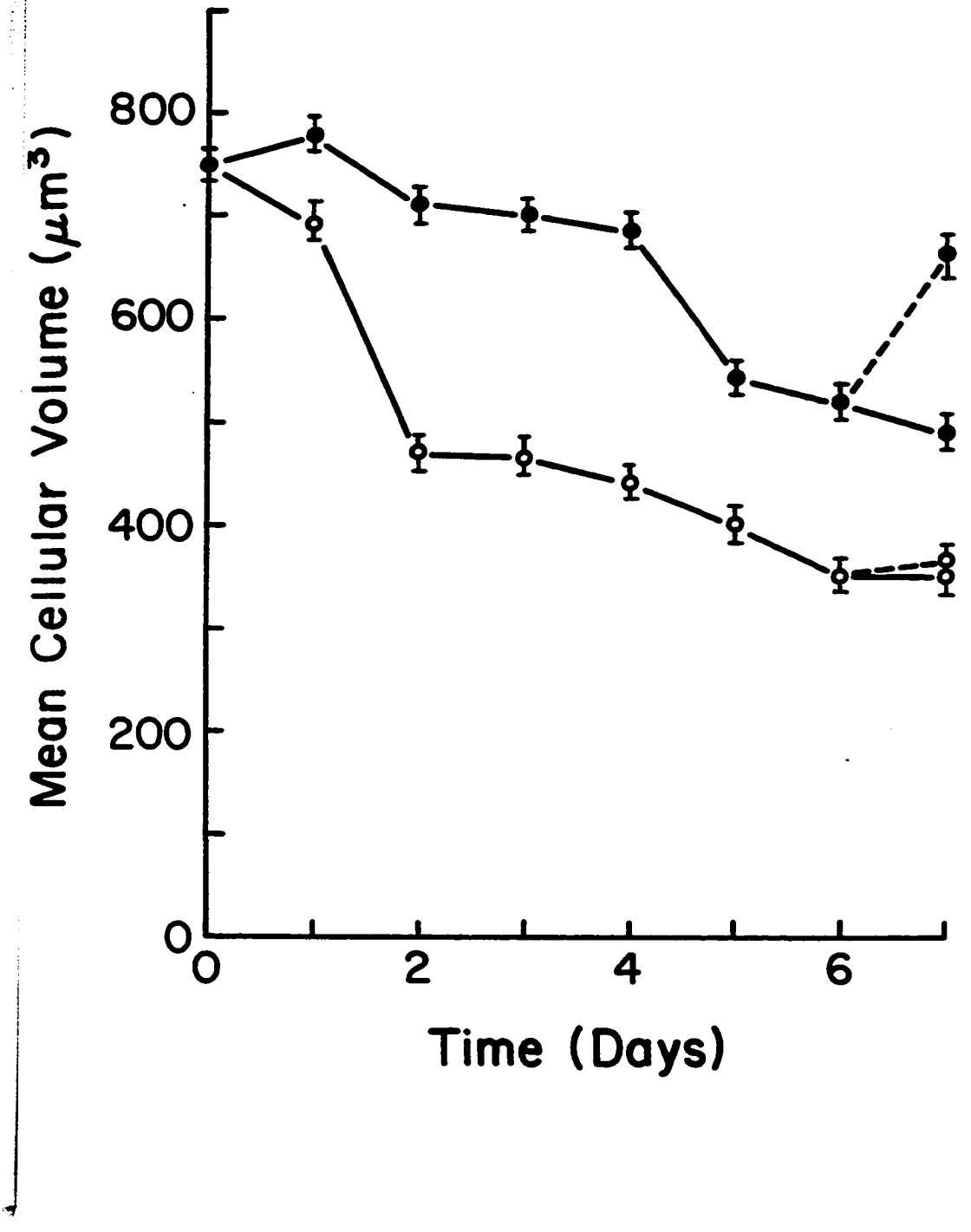
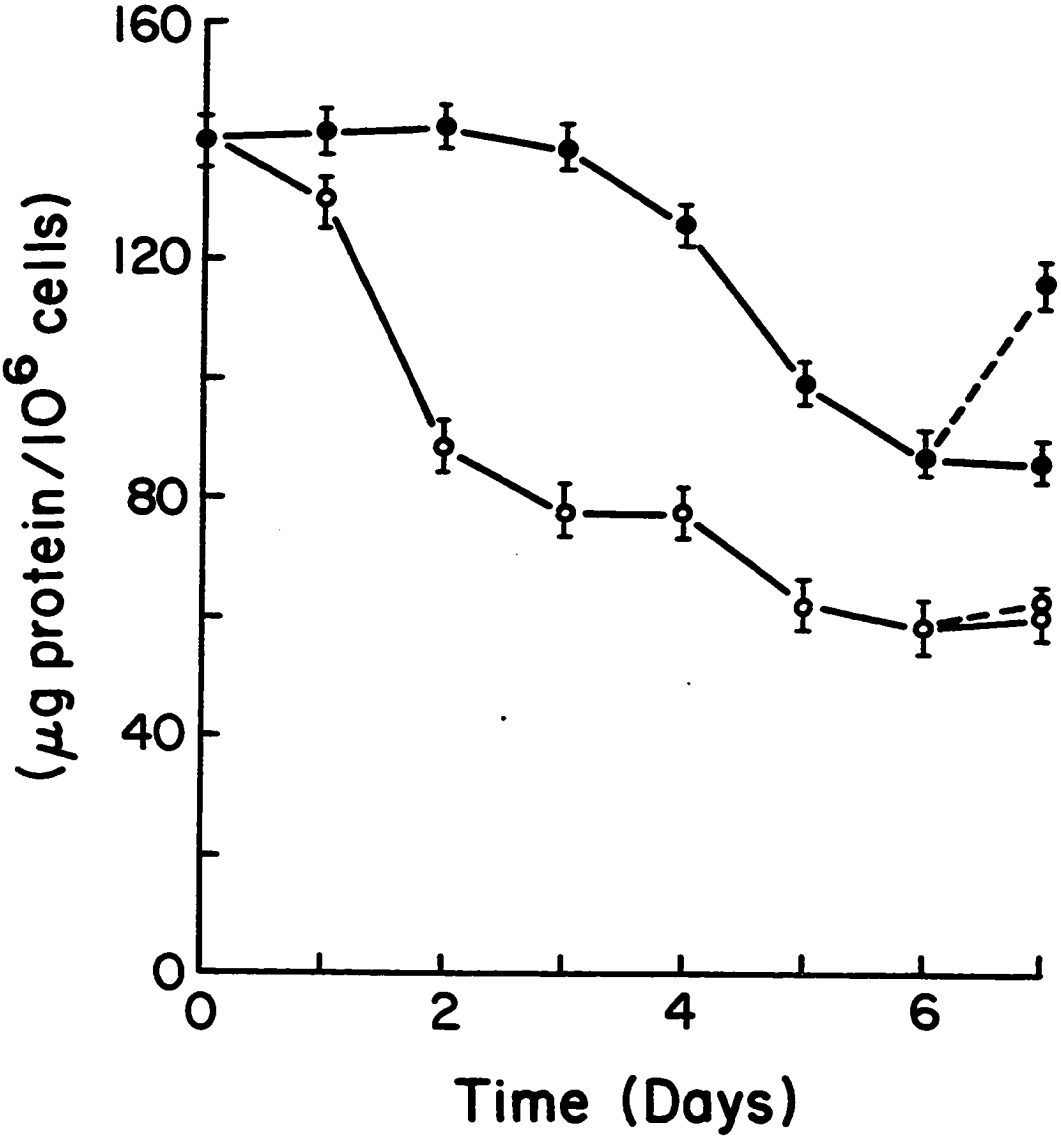


Figure 5. Cellular protein content of HL-60 cells during DMSO-induced maturation. Cells were treated with 1.2% DMSO (v/v) (o) untreated (●). At each time point, cells were lysed, and total protein content determined as described in "Materials and Methods." After 6 days, an aliquot of cells was placed in fresh medium, and a separate determination made one day later (----). Each point represents the mean ± standard error of the mean of three separate experiments each done in duplicate.



Phosphoaminoacid distribution with granulocytic differentation of HL-60 cells. Phosphotyrosine (P-tyr) generally makes up less than 1% of cellular protein phosphoaminoacids. Untreated HL-60 cells have 1.5% P-tyr among their phosphoaminoacids, with the remainder consisting of phosphoserine (P-Ser) and phosphothreonine (P-thr) in a 9:1 ratio (Table 1). Upon maturation induced by either DMSO or RA there is a 5-to 8-fold decrease in P-tyr with no significant change in the other phosphorylated amino acids. When untreated cells reach plateau phase, they exhibit a small decrease in relative P-tyr content compared to log phase cells, though not nearly so dramatic as the differentiating cells.

Characterization of HL-60 tyrosine kinase. Intracellular levels of phosphotyrosine are regulated by two enzyme activities: tyrosine kinase and protein phosphotyrosine phsophatase. As a first step in understanding the control of tyrosine phosphorylation, experiements were performed to characterize each of these activities.

Tyrosine kinase activity was measured by the incorporation of ^{32}P from [γ-^{32}P] ATP into specific peptide substrates. These substrates (glutamic acid: tyrosine, 4:1 (GT); glutamic acid: alanine: tyrosine, 6:3:1 (GAT), and angiotensin II) contain tyrosine as the only hydroxylated amino acid, so that any incorporation of phosphate

TABLE 1

Phosphoaminoacid Content of HL-60 Cells During Granulocytic Differentiation

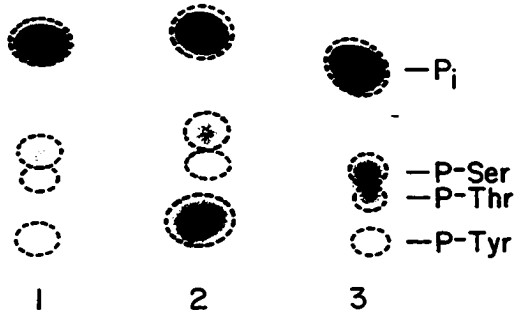
Treatment	P-Tyr	P-Ser	P-Thr
None (log phase)	1.5 ± 0.2	88.2 ± 0.9	10.3 ± 0.4
None (plateau)	1.3 ± 0.2	88.4 ± 0.9	10.3 ± 0.4
DMSO (1.2% v/v, for 6 days)	0.2 ± 0.1	89.4 ± 1.1	10.4 ± 0.4
RA (10^{-6}M for 6 days)	0.3 ± 0.1	89.2 ± 1.0	10.5 ± 0.3

Percentages of phosphorylated amino acids represented by phosphotyrosine (P-Tyr), phosphoserine (P-Ser), and phosphothreonine (P-Thr). HL-60 cells were treated as described and the phosphoaminoacid distribution was determined as described in "Materials and Methods". Values represent the mean ± standard deviation of 3 separate determinations.

represents tyrosine phosphorylation, rather than serine or threonine phosphorylation. The phosphorylation of histone (which contains serine, threonine, and tyrosine) was used to assess total kinase activity. GAT and GT are random polymers of glutamic acid and tyrosein (GT) or glutamic acid, alanine, and tyrosine (GAT) which are synthesized chemically. GAT has an average MW of 25,000; GT has an average MW of 28,000. The phosphorylation of histone II (which contains serine, threonine, and tyrosine) was used to assess total kinase activity. (The histone preparation used is a mixture of the histone proteins, and is prepared by the extraction of calf thymus in 1M NaCl, precipation in water, acid extraction, and reprecipitation with alcohol. It is designated type II by its supplier (Sigma), although this is unrelated to the classical histone nomenclature of Luck and coworkers (138a)).

In determining the tyrosine kinase activity of a given cellular fraction, the incorporation of ^{32}P was measured in the presence and absence of substrate. In the absence of substrate, some phosphate incorporation would be expected via the phosphorylation of endogenous proteins. As shown in Figure 6, lane 1, thin layer electrophoresis of the products of a phosphorylation reaction in the absence of added substrate reveals that nearly all of the phosphate incorporation is in P-ser, with a small amount in P-thr, and none detectable as P-tyr. Upon addition of a substrate

Figure 6. Phosphoaminoacid analysis of *in vitro* phosphorylation reaction products. HL-60 particulate fractions were incubated in tyrosine kinase buffer with 20 uM [γ-^{32}P) ATP (3 Ci/mmol) for 10 minutes at 22^{o}C with no substrate (lane 1), 1 mg/ml GAT (lane 2), or 3 mg/ml histone II (lane 3). The reaction products were hydrolyzed with HCl, and phosphoaminoacids separated as described in "Materials and Methods." Abbreviations: P_i, inorganic phospate; P-ser, phosphoserine; P-thr, phosphothreonine; and P-tyr, phosphotyrosine.



containing only tyrosine (GAT; lane 2), a large amount of P-tyr was detected (greater than 9 times that in P-ser and P-thr), but no change in the amount of the other two phosphoaminoacids. This indicated that the addition of substrate had no effect on endogenous phosphorylation, and that the difference in phosphate incorporation between samples containing and lacking the tyrosine-containing substrate represented net tyrosine phosphorylation. When a substrate containing all of the hydroxylated amino acids (histone type II) was used (lane 3), serine and to a lesser extent threonine phosphorylation was observed, though no P-tyr was detected. This likely reflects the large excess (in quantity and activity) of serine/threonine kinases relative to tyrosine kinases in cell extracts.

Given the ability of this system to detect net tyrosine kinase activity, a more convenient assay system was sought. The filter paper method of Corbin and Reimann (135), originally devised for assaying cyclic AMP-dependent protein kinases, was modified for this assay. This technique exploits the fact that macromolecules in solution spotted onto a heavy fiber filter paper (e.g., Whatman 3MM) will precipitate onto the fibers upon exposure to 10% TCA. This property allows the filters to be washed extensively to remove excess ATP. It was found that adding 10 mM sodium pyrophosphate to the TCA reduced non-specific binding (likely through an ionic effect), and that 5 wash cycles

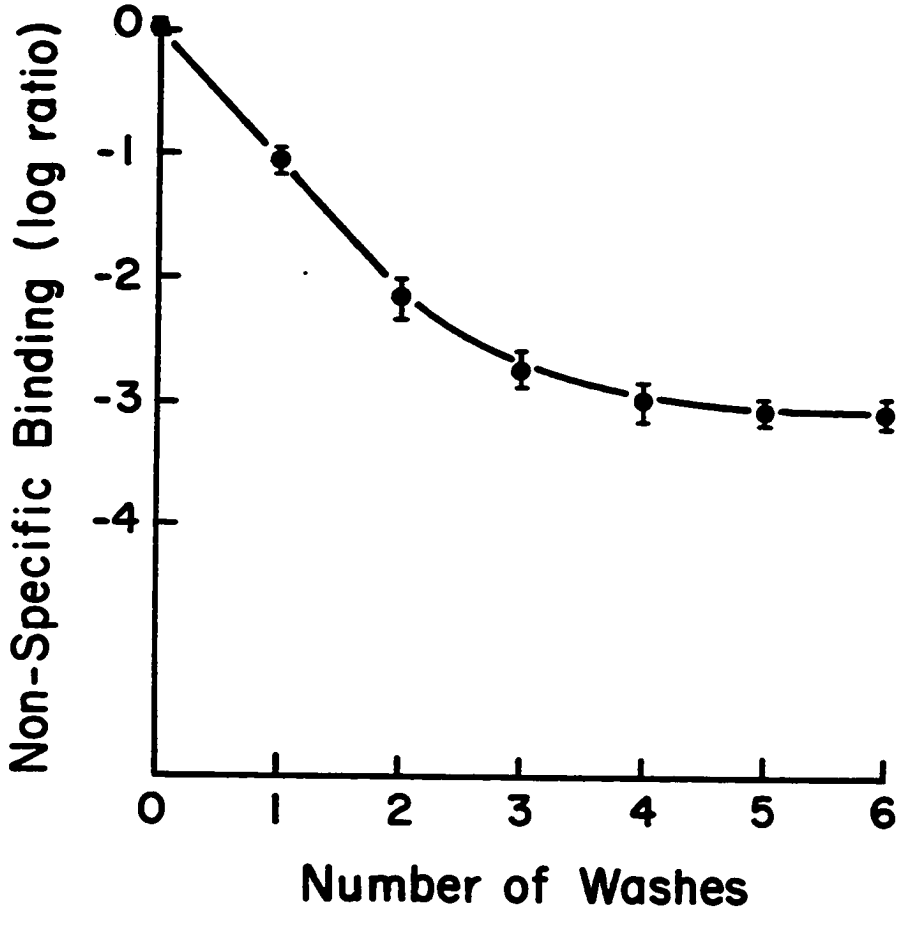
provided a minimum of non-specific binding (Figure 7). Thus this filter paper assay was employed to quantitate tyrosine kinase activity, with net activity expressed as the difference between samples containing and lacking the substrate.

As this system measures total incorporation of ^{32}P into TCA-insoluble macromolecules, the possibility of incorporation by mechanisms other than transfer of the γ-phosphate of ATP was considered. Two potential mechanisms include high affinity binding of ATP to the substrate, and inter-nucleotide phosphate transfer followed by adenylation of the substrate. To address this question, an equimolar amount of [α-^{32}P] ATP was substituted for [γ-^{32}P] ATP in the reaction mixture. The complete absence of specific incorporation into substrate abolished these possibilities.

HL-60 cells were fractionated (as described in "Materials and Methods") by disruption via sonication followed by low speed centrifugation to remove nuclei and unbroken cells, and a high speed centrifugation (30,000 x g for 30 minutes) to separate the particulate from the soluble fractions. The particulate fraction was then solubilized in 0.5% Nonidet-P40. Greater than 90% of the recovered tyrosine kinase was recovered in the particulate fraction, upon which all of the characterizations were performed.

Employing GAT as a substrate, tyrosine kinase activity of HL-60 particulate fractions was linear with respect to

Figure 7. Non-specific binding of ^{32}P to filters as a function of wash number. Whatman 3MM filter paper squares to which 0.1 uCi (γ-^{32}P) ATP was added, were dried, and washed as described in "Materials and Methods." The fraction of radioactivity remaining on each filter was determined after each wash. Each point represents the mean ± standatd error of the mean of two separate experiments each done in duplicate.



protein concentration through approximately 25 ug/assay mixture, and plateaued at about 50 ug/mixture (reaction velocity of 0.4 pmol/min) (Figure 8). Tyrosine kinase activity was temperature dependent, being minimal but detectable at $0^{o}C$ and increasing through $37^{o}C$; the reaction was also initially linear with time at all temperatures (Figure 9). In order to confirm this increasing tyrosine phosphorylation with time, aliquots of the reaction mixture with and without substrate were removed and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 10). In the presence of substrate (lanes 5-8) there is a time dependent incorporation of ^{32}P into the 20,000 to 30,000 MW range in which the substrate would migrate; no such labeling is evident in the lanes lacking substrate (lanes 1-4).

ATP can serve as phosphate donor for the tyrosine kinase reaction in a concentration dependent manner (Figure 11); phosphorylation is linear with ATP to a concentration of about 50 uM, at which point it rapidly reaches plateau. The apparent K_m for ATP is approximately 22 uM. Although the question of alternate phosphate donors was not addressed directly, the ability of nucleotides to compete with ATP was examined (Table 2). The addition of 100-fold excess unlabeled ATP (relative to the labeled nucleotide) led to nearly complete abolition of ^{32}P incorporation. Similarly, such excess of ADP, AMP, and adenosine also diminished

Figure 8. Tyrosine kinase activity as a function of HL-60 particulate fraction protein content. Tyrosine kinase assays were performed as described in "Materials and Methods" in total volume of 50 ul of tyrosine buffer containing 25 uM [γ-^{32}P] ATP (3 Ci/mmol), 1 mg/ml GAT, and the indicated amount of protein, at 22^{o}C. Each point represents the mean ± standard error of the mean of 3 separate determinations each done in duplicate.

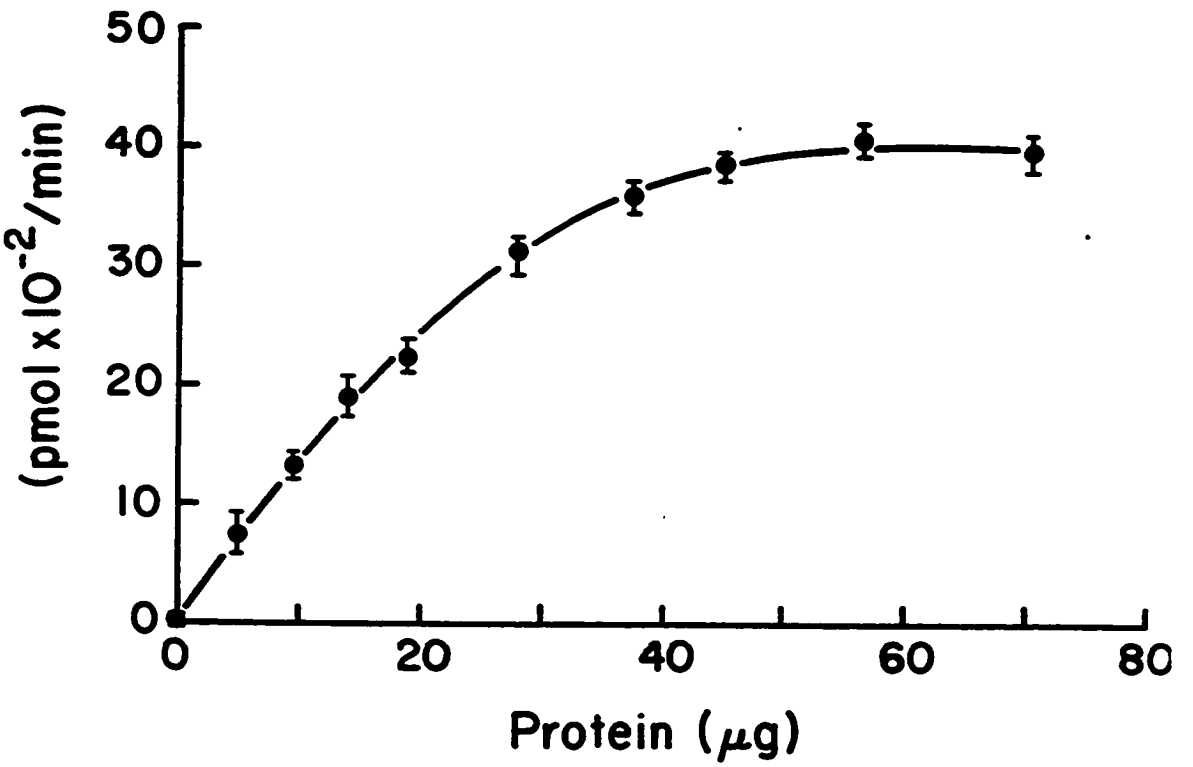


Figure 9. Temperature dependence of HL-60 tyrosine kinase activity. Tyrosine kinase activity was determined at 0 °C (□), 23 °C (△), and 37 °C (●), using 20 ug of particulate fraction protein and 25 uM ATP. Each point represents the mean ± standard error of the mean of three separate experiments each done in duplicate.

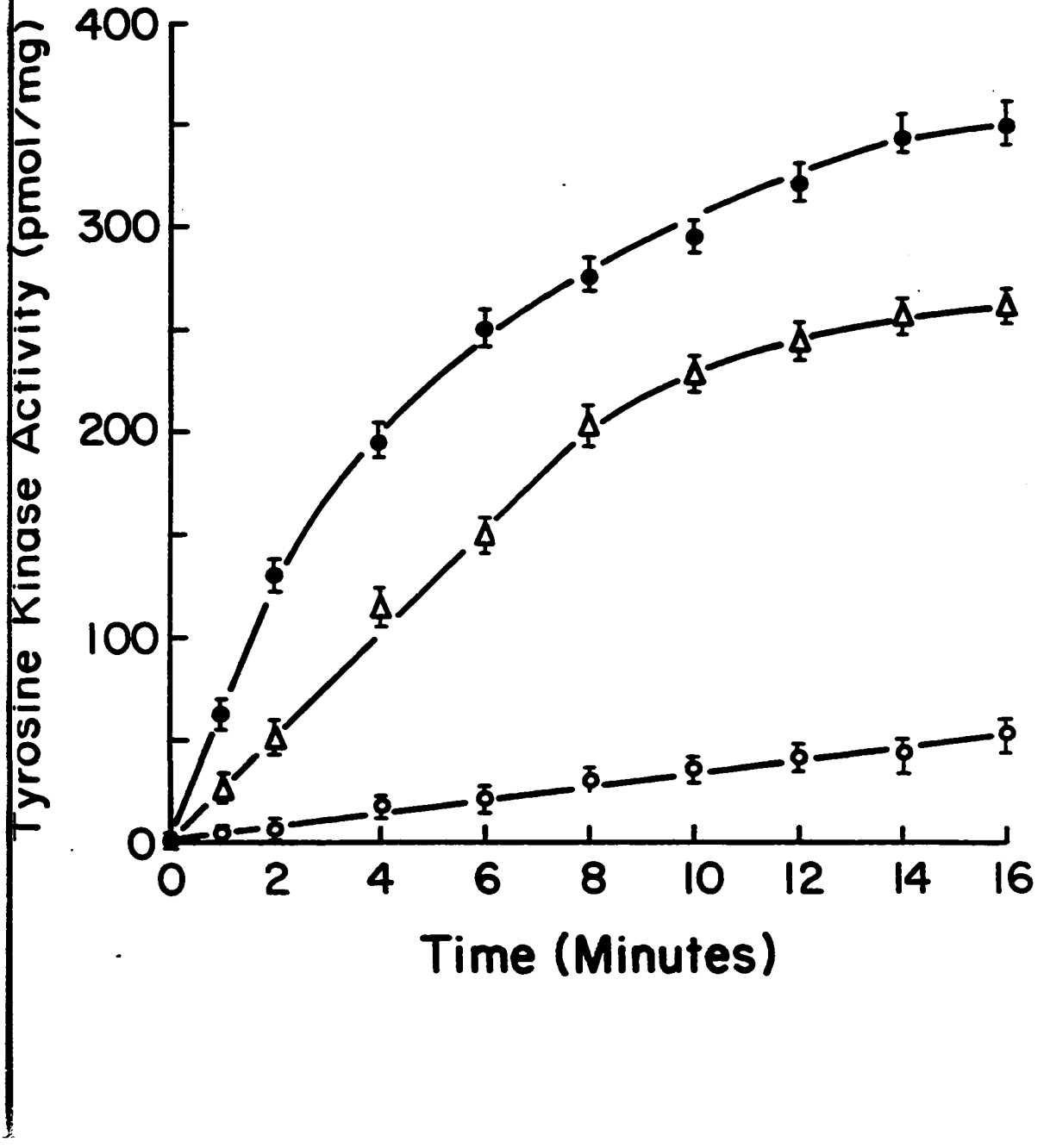


Figure 10. SDS-PAGE of tyrosine kinase reaction products. Tyrosine kinase reactions were allowed to proceed for 1 minute (lanes 1 and 5), 5 minutes (lanes 2 and 6), 10 minutes (lanes 3 and 7), and 15 minutes (lanes 4 and 8) in the absence (lanes 1 through 4) and presence (lanes 5 through 8) of 1 mg/ml GAT. Reactions were performed at 23 °C with 20 ug of HL-60 particulate fraction and 25 uM ATP. Reactions were terminated by boiling mixtures in 1% (w/v) SDS and 5 uM 2-mercaptoethanol, and the products resolved on a 10% polyacrylamide gel with a 4% stacking gel. The gel was fixed and dried, and exposed to x-ray film for 72 hours.

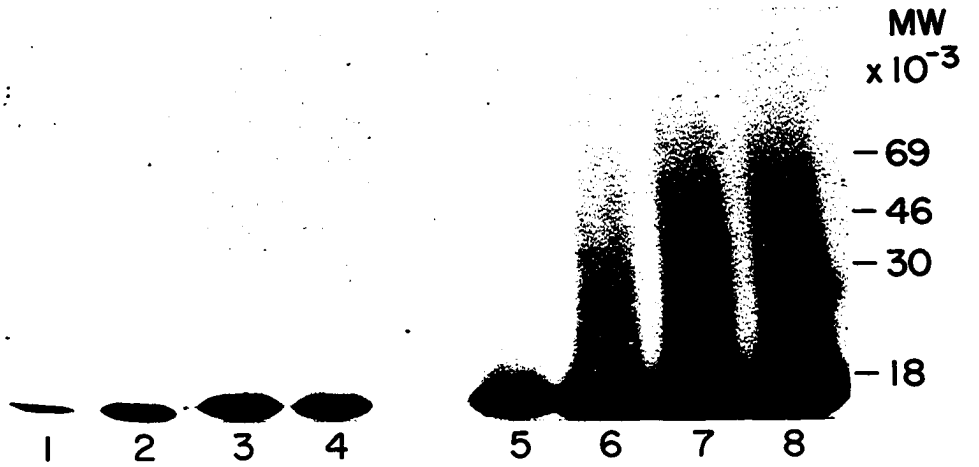


Figure 11. HL-60 tyrosine kinase activity as a function of ATP concentration. Tyrosine kinase assays were performed at 23°C using 20 ug of particulate fraction protein and the ATP concentrations indicated. Each point represents the mean ± standard error of the mean of 3 experiments each done in duplicate.

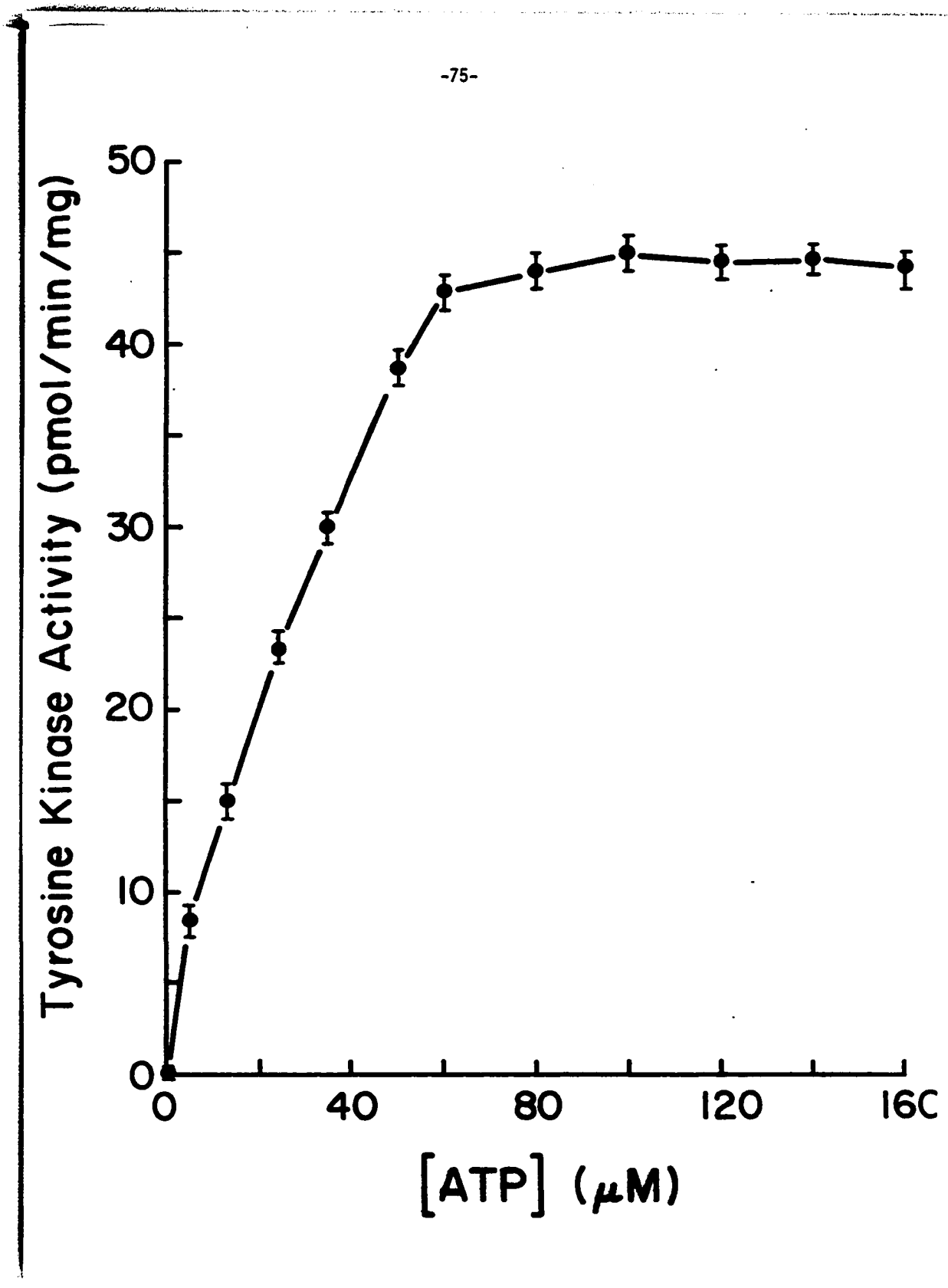


TABLE 2

The Effect of Nucleotides on Tyrosine Kinase Activity in HL-60 Cells

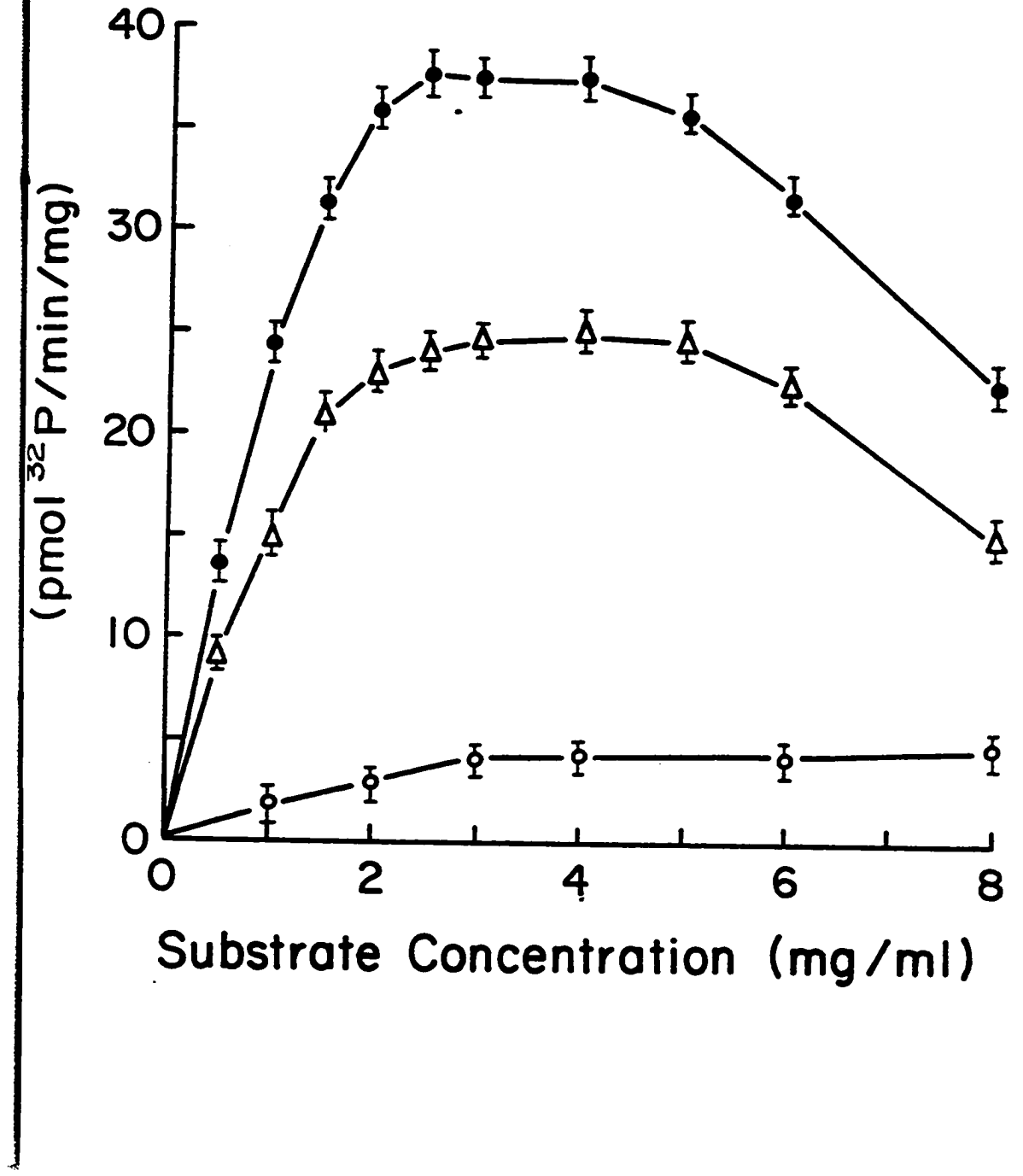
Nucleotide Added	% Control Activity
---	100
ATP	4.6
ADP	1.2
AMP	3.6
Adenosine	9.7
GTP	25.2
GDP	33.5
GMP	83.5
dGTP	23.0
dGuanosine	45.9
UTP	62.2
CTP	56.8
CDP	88.4

A 100-fold excess of unlabeled nucleotide (1 mM) was added to the standard tyrosine kinase assay and the activity was measured. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

labeling by greater than 90%. 100-fold excess of GTP (or dGTP) abolished 75% of incorporation; GMP and deoxyguanosine caused somewhat less inhibition. A number of pyrimidine di- and tri- phosphates had intermediate effects.

Among substrates, either GT or GAT could serve as recipients in the phosphorylation assay (Figure 12), although the initial velocity is approximately 1.6-fold greater for the GT (on a weight basis). GAT has an average MW of 25,000 with a degree of polymerization (i.e., average chain length) of 190. It consists of 8% tyrosine, and thus a 1 mg/ml solution of GAT has a tyrosine concentration of 0.61 mM. GT has an average MW of 28,000, a degree of polymerization of 160, and a tyrosine content of 17%, such that a 1 mg/ml solution of GT has a tyrosine concentration of 0.97 mM. Thus, expressed in terms of tyrosine concentration (which is the ultimate substrate), the initial velocities are very similar for the two polymers. Expressed in terms of tyrosine concentration, the apparent k_m for the two substrates does vary, being 0.49 mM for GAT and 0.73 mM for GT. At higher substrate concentrations there is a decrease in activity shown by both polymers. In order to determine whether the HL-60 tyrosine kinase could phosphorylate other substrates, the basic octapeptide angiotensin II was employed. This peptide was phosphorylated, although at a slower rate than the random polymers. Angiotensin II has a formula weight of 1218, and

Figure 12. Effect of substrate on HL-60 tyrosine kinase activity. Assays were performed at 22° using 30 ug particulate fraction protein, 25 uM ATP, and either GT (●), GAT (▲), or angiotensin (○) at the indicated concentrations. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.



one tyrosine residue per molecule; thus a 1 mg/ml solution has a tyrosine concentration of 0.82 mM. The apparent k_m for angiotensin II was approximately 1.1 mM; no inhibition at higher concentrations was seen.

The HL-60 tyrosine kinase activity had a pH optimum of about 6.5, with a gradual falloff at both higher and lower pH levels (Figure 13). The effects of pH values greater than 7.5 could not be assessed because of the precipitation of Mn^{2+} under alkaline conditions. No significant difference in pH response was seen employing three different buffers: Hepes, Tris, and morpholino propane sulfonic acid (MOPS).

Most ATP-dependent enzymes require a divalent cation -- generally either Mn^{2+} or Mg^{2+} -- as a co-factor. HL-60 tyrosine kinase activity shows minimal (though detectable) activity in the absence of an added cation (Figure 14). The addition of Mg^{2+} leads to increased activity up to about 15 mM, after which it remains constant. Mn^{2+} causes increased activity to about 12 mM; at concentrations above 25 mM, however, there is decreased activity such that at 50 mM the kinase activity is completely abolished. The combination of 12 mM Mn^{2+} and 15 mM Mg^{2+} does not increase the level of phosphorylation above that seen with Mn^{2+} alone. Thus, all further experiments were performed in the presence of 12 mM Mn^{2+} and no Mg^{2+}.

The addition of Zn^{2+} (10 uM) in the presence of 12 mM

Figure 13. pH dependence of HL-60 tyrosine kinase activity. Assays were performed at 22 °C using 20 ug of particulate fraction protein, 25 uM ATP, and 1 mg/ml GAT at the indicated pH values in Hepes buffer. Each point represents the mean ± standard error of the mean of three separate experiments each done in duplicate.

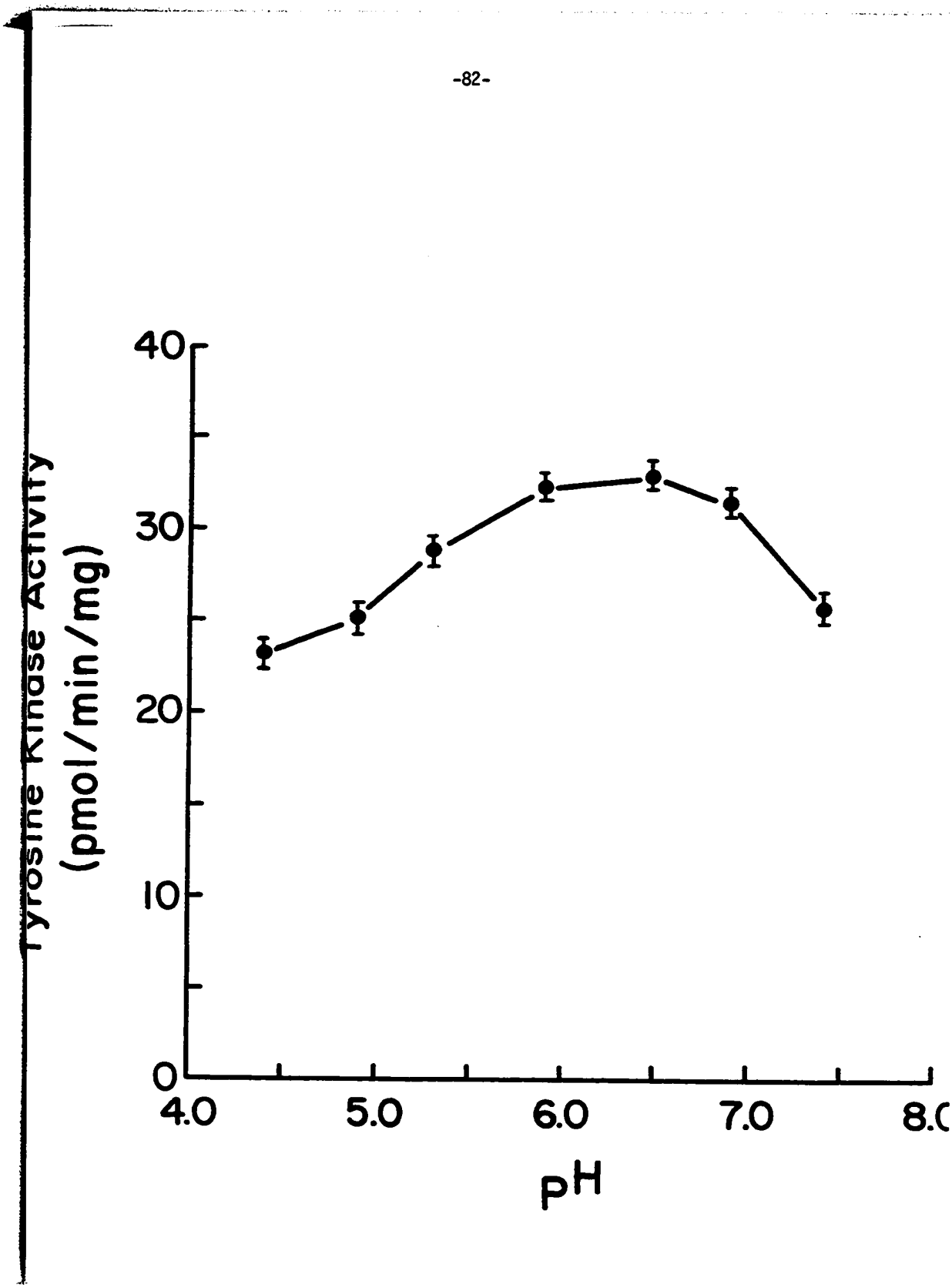
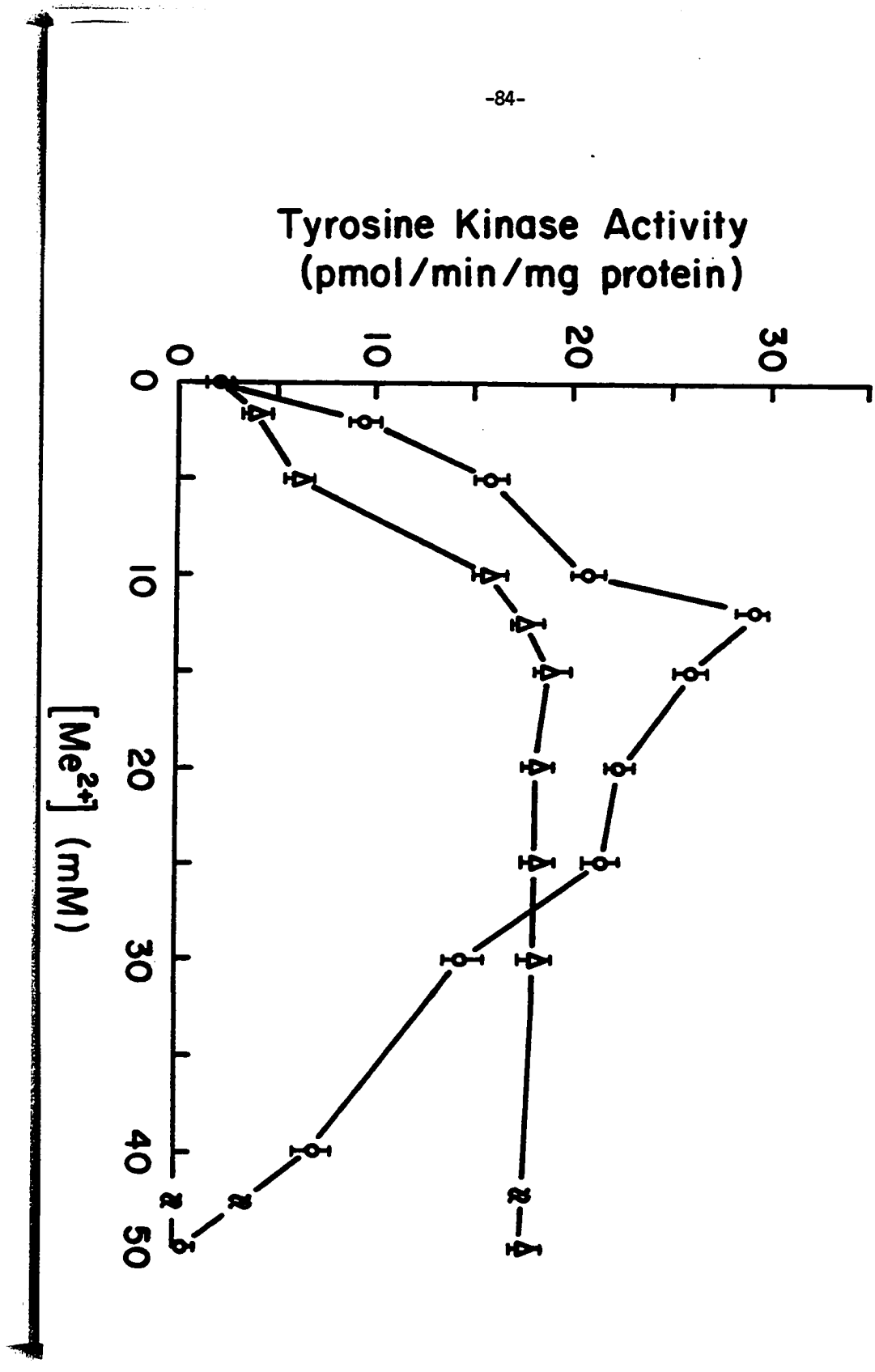


Figure 14. Effect of Mg^{2+} and Mn^{2+} on HL-60 tyrosine kinase activity. Assays were performed at 23 °C using 15 ug of particulate fraction protein, 1 mg/ml GAT, and Mg^{2+} (Δ) or Mn^{2+} (O) concentrations as shown. Each point represents the mean ± standard error of the mean of three separate experiments each done in duplicate.



Mn^{2+} led to a 20% increase in kinase activity (Figure 15) and was also included in the assay mixtures. Higher concentrations of Zn^{2+} led to a precipitous fall in activity.

The effect of ionic strength on the kinase activity was examined next. Up to 100 mM NaCl had no influence on activity; at 500 mM, 71% inhibition was seen and at 1 M, 79% of the kinase activity was abolished. LiCl reduced activity by 67% at 100 mM.

Although tryosine kinases have been found to be cAMP-independent, the effect of this cyclic nucleotide on the HL-60 activity was assessed. At concentrations up to 10^{-4} M, no effect was discernible (Table 3B). Similarly stimulators of protein kinase C were also assessed. The phorbol ester 12-0-tetradecanoyl phorbol 13-acetate (TPA) showed no effect up to 10^{-5} M; Ca^{2+} had no effect up to 10^{-3} M. at 10^{-2} M, however, activity was inhibited 40%; at 10^{-1} M, it was totally abolished (Table 3A).

Given that a number of growth factor receptors possess tyrosine kinase activity, the question arose as to whether the activity detected in HL-60 cells was that of a receptor. Two possibilities were examined: the insulin receptor and the EGF receptor. Neither insulin (up to 10^{-6} M) nor EGF (up to 10^{-6} M) had any effect (stimulatory or inhibitory) on the HL-60 tyrosine kinase activity (Table 3B).

It has been suggested that tyrosine kinases also may

Figure 15. Effect of Zn^{2+} on HL-60 tyrosine kinase activity. Assays were performed at 23 °C using 1 mg/ml GAT and Zn^{2+} concentrations as shown. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

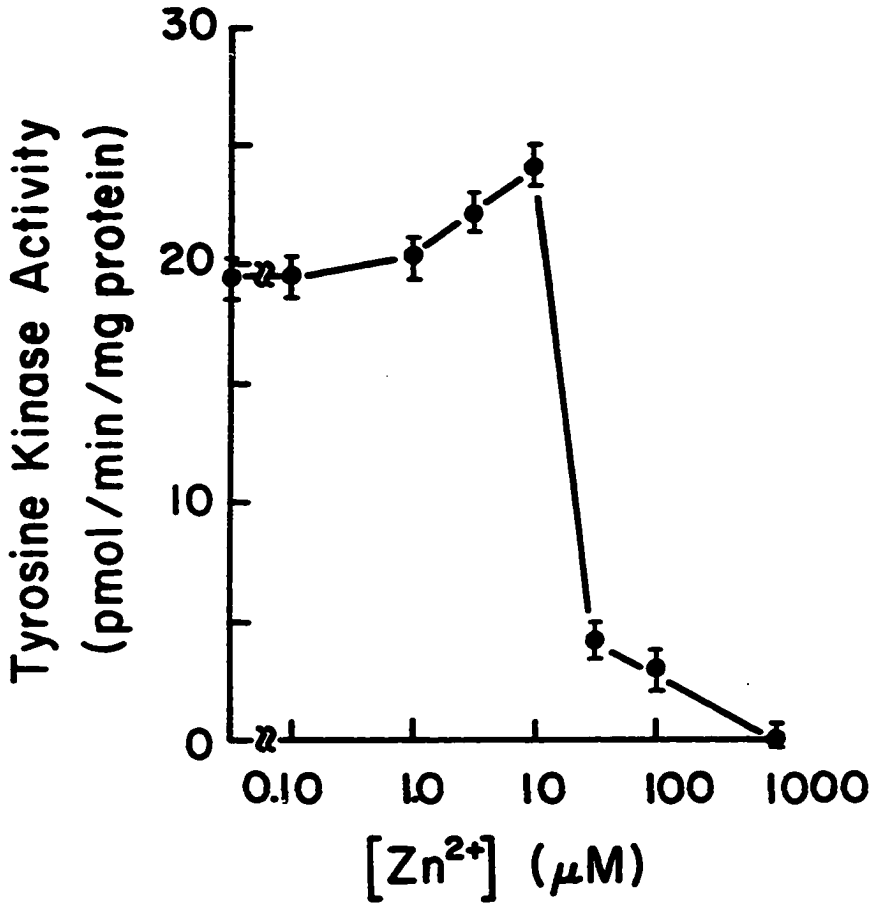


TABLE 3

Effects of Various Compounds on HL-60 Tyrosine Kinase Activity

A. Agents which inhibit activity:

Compound	Concentration	% Activity
None	---	100
NaCl	0.01 mM	98
	0.1	96
	0.5	29
	1.0	21
LiCl	1.0 mM	97
	10.0	73
	100.0	33
$CaCl_2$	1.0 mM	102
	10.0	59
	100.0	0
Heparin	1 µg/ml	104
	5	80
	10	71
	50	74
TLCK	1 mM	102
	5	51
	10	32
	20	35

B. Agents with no effect:

Compound	Maximum concentration tested
Insulin	10^{-6} M
EGF	10^{-6} M
cAMP	10^{-4} M
TPA	10^{-5} M
Phosphatidyl inositol	200 µg/ml

Agents were added to the standard tyrosine kinase assay at indicated concentrations, and activity was measured. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

phosphorylate glycolipids, particulary phosphatidyl inositol (PI). To determine whether the HL-60 tyrosine kinase can also phosphorylate PI, the glycolipid was added to the kinase mixture to see whether tyrosine phosphorylation would be inhibited. At concentrations of up to 200 ug/ml, PI had no effect on activity (Table 3B).

The trypsin and papain inhibitor N-p-tosyl-L-lysine chloromethyl ketone (TLCK) was also examined for activity against the HL-60 tyrosine kinase. A dose dependent inhibition was seen, up to 68% inhibition at 10 mM (Table 3). As high concentrations of the polymer substrates appear to inhibit activity, the possibility arose that the large concentration of negatively charged groups (the γ-carboxyl moieties from the glutamic acid) might be inhibiting the activity. To examine this possibility, heparin, a negatively charged polymer of comparable MW was employed. As heparin cannot be phosphorylated, the effect of the polyanion on the HL-60 tyrosine kinase activity could be assesses. Heparin caused a small inhibition, maximally of 29% at a concentration of 10 ug/ml (Table 3A).

It has been suggested that the orthovanadate ion (VO_4^{-3}) can directly stimulate a number of tyrosine kinases including the insulin receptor (82) and others (83). Thus, the effect of this ion on the HL-60 tyrosine kinase was evaluated. At concentrations up to 10^{-4} M, no effect was discerned; at 10^{-3} M, little or no inhibition of activity was

observed (Figure 16). In addition to directly stimulating a number of kinases, vanadate is also a potent inhibitor of known phosphotyrosine phosphatases at micromolar concentrations (80). The fact that vanadate, at these concentrations, fails to stimulate incorporation of ^{32}P into phosphotyrosine by HL-60 tyrosine kinase activity also suggests that phosphotyrosine phosphatase activity is not a significant factor during this assay. To further examine this question, a tyrosine kinase assay was conducted in which 100-fold unlabeled ATP was added after 8 minutes of incorporation (Figure 17). This prevented the further incorporation of ^{32}P into the substrate. The radiosactivity remaining in the substrate was then assessed at 5 minute intervals thereafter. As can be seen in Figure 17 no decrement in radioactivity ensued, further indicating that phosphotyrosine phosphatase activity was not an important factor in the HL-60 tyrosine kinase assay.

The effect of a number of detergents on HL-60 tyrosine kinase activity was examined next. Tyrosine kinase activity could be recovered from HL-60 particulate fractions in the absence of any detergent (Table 4), though extraction with either 0.1% (v/v) Triton X-100 or 0.1% (v/v) Nonidet P-40 led to a 2.5-fold enhancement of recoverable activity. The same concentration of sodium desoxycholate had no effect. In the reaction mixture itself, 0.5% (v/v) of either Triton X-100 or Nonidet P-40 led to a maximal 1.6- to 1.9-fold increase in activity.

Figure 16. Effect of VO_4^{3-} on tyrosine kinase activity. Assays were performed at 22 °C using 1 mg/ml GAT with (Δ) or without (o) 1 mM Na_3VO_4. The 6 min time point was the only one demonstrating a significant difference ($p<0.05$) as measured by the student's t-test (two-tailed). Each point represents the mean ± standard error of the mean of three separate experiments each done in duplicate.

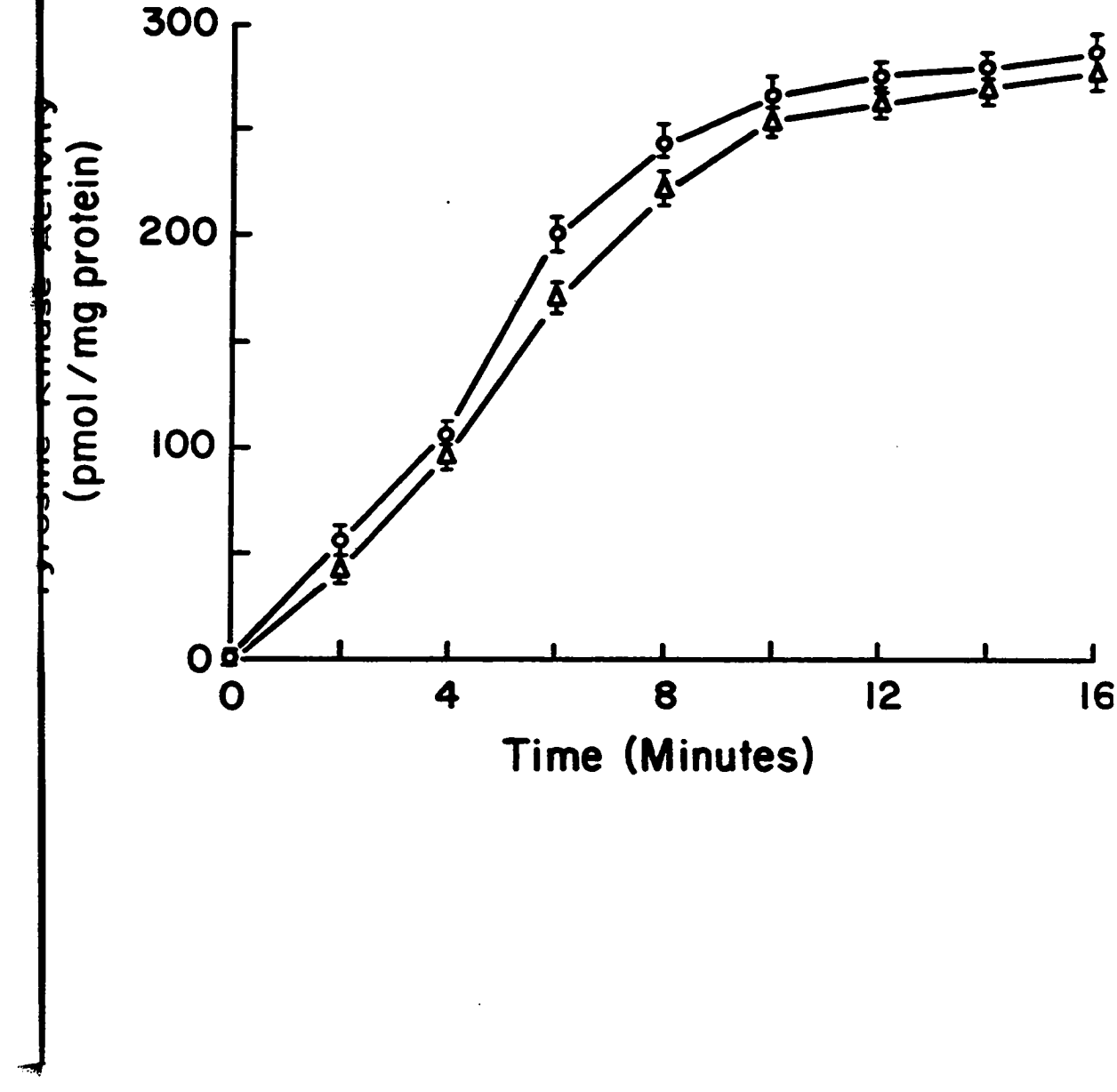


Figure 17. Effect of unlabeled ATP on ^{32}P incorporation into substrate. A tyrosine kinase assay was allowed to progress for 8 minutes at 22 °C, using 15 ug of protein, 25 uM ATP (3 Ci/mmol), and 1 mg/ml GAT. At this point (arrow), 100-fold excess unlabled ATP (2.5 mM) was added to prevent further incorporation of the isotope, and the amount of label in the substrate was determined at subsequent time points. Each point represents the mean ± standard error of the mean of two separate experiments each done in duplicate.

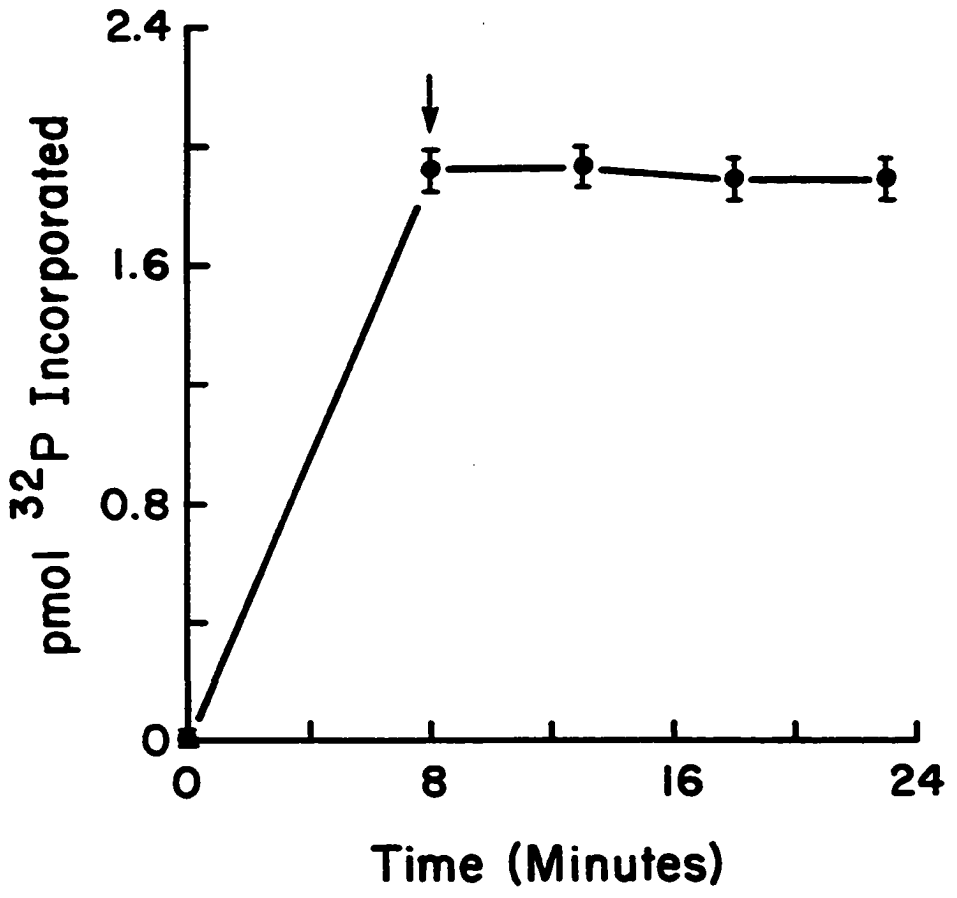


TABLE 4

The Effects of Detergents on HL-60 Tyrosine Kinase Activity

A. Effect on extraction:

Detergent	Activity (% of control)
None	100
0.1% Nonidet P-40	248
1.0% Nonidet P-40	148
0.1% Triton X-100	226
1.0% Triton X-100	165
0.1% Sodium desoxycholate	106
1.0% Sodium desoxycholate	87

B. Effect on assay:

Detergent	Activity (% of control)
None	100
0.1% Nonidet P-40	88
0.5% Nonidet P-40	159
1.0% Nonidet P-40	127
0.1% Triton X-100	140
0.5% Triton X-100	191
1.0% Triton X-100	138

Various detergents were used either in the extraction of the particulate fraction (A) or in the tyrosine kinase assay itself (B), and activity was measured. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

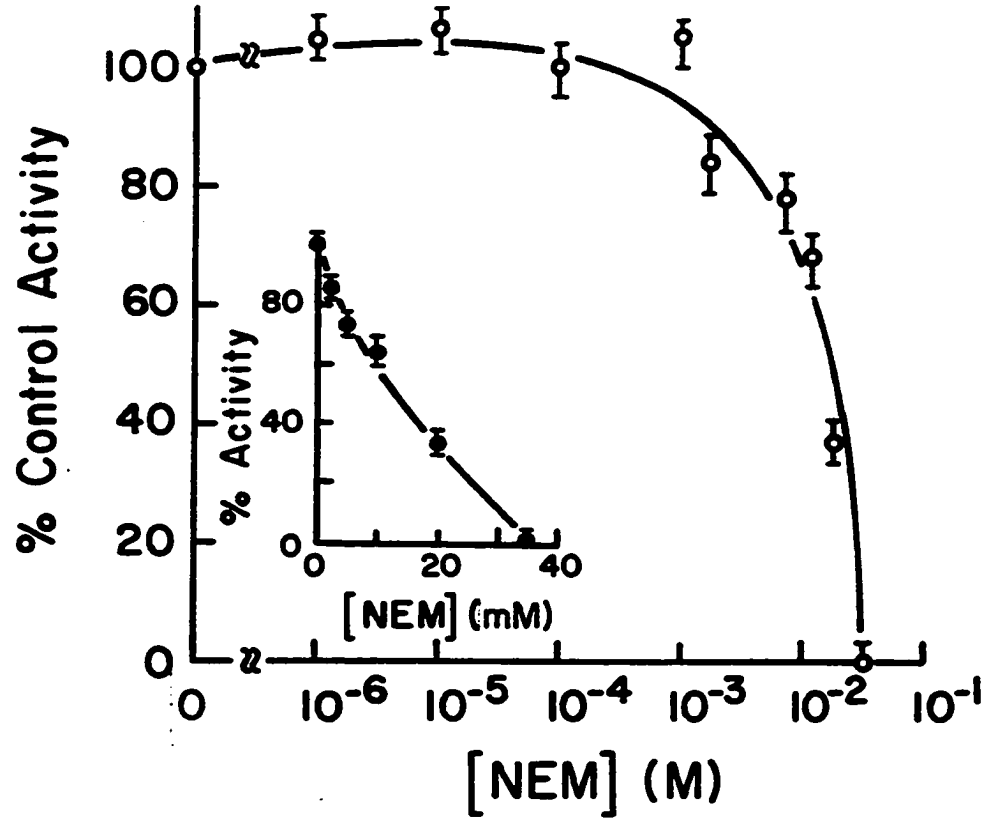
N-Ethyl maleimide (NEM), a compound which inactivates sulfhydryl groups, was assessed for its effect on the tyrosine kinase activity. Activity was inhibited by NEM in a concentration-dependent and time-dependent manner (Figure 18).

The effects of a number of small molecules on HL-60 tyrosine kinase activity was assessed next (Table 5). Up to 5 mM of free P-ser or P-thr had no effect on activity. P-tyr had no effect up to a concentration of 0.1 mM; at 1.0 mM, however, 94% of activity was abolished. At 1.0 mM, free tyrosine inhibited 76% of the activity. Disodium phenylphosphate, a P-tyr analog, caused maximal inhibition at 10^{-4} M (26%). Amiloride, an inhibitor of Na^+/H^+ exchange, has also been found to inhibit the EGF receptor tyrosine kinase (139). In assays with the HL-60 tyrosine kinase, however, amiloride had no effect at concentrations up to 1 mM.

Quercetin, a bioflavinoid compound, has been found to inhibit tyrosine kinases from rat lung (140), RSV (141), and a mouse mammary tumor (142). With the HL-60 tyrosine kinase, quercetin inhibited activity by 36% at 10^{-4} M, and by 96% at 10^{-3} M. Levamisole, an immunomodulator with some clinical success against human tumors (143), was without effect on HL-60 tyrosine kinase activity up to concentrations of 10 mM.

Heating of HL-60 particulate fraction protein (56 ^{o}C

Figure 18. Panel A. Effect of N-ethylmaleimide (NEM) on tyrosine kinase activity. Fifteen micrograms of particulate fraction protein was incubated with various concentrations of NEM for 15 minutes at 23 °C, after which all unreacted NEM was scavenged by the addition of 30 mM dithiothreitol. Assays were then performed as before; dithiothreitol alone had no effect on activity. Panel B. Particulate fraction protein was treated with 30 mM NEM for various times before addition of dithiothreitol, and assays performed as in Panel A. Each point in both Panels A and B represents the mean ± standard error of the mean of three distinct experiments each done in duplicate.



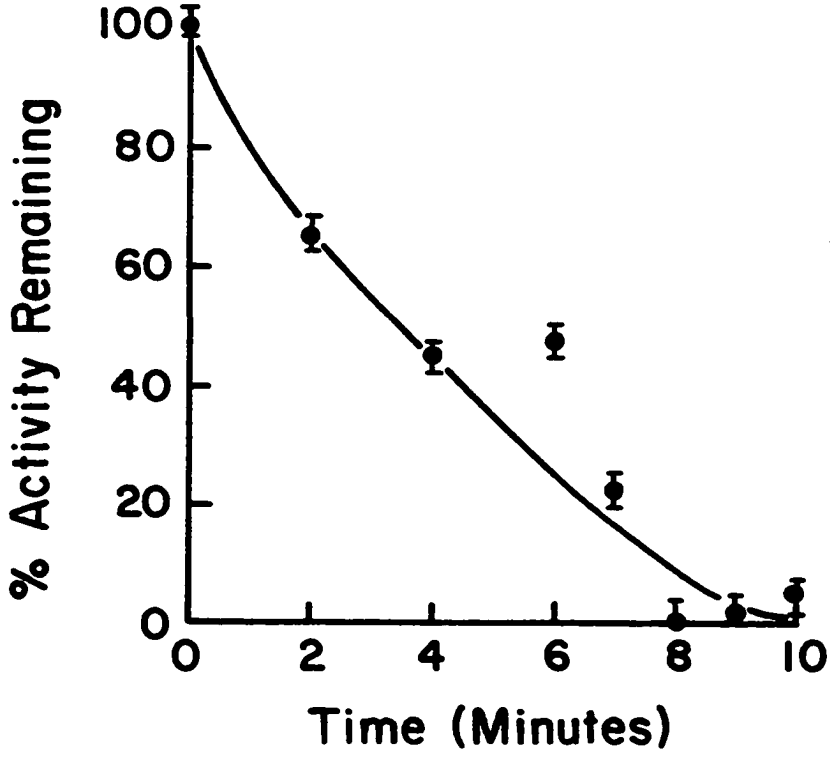


TABLE 5

Effect of Small Molecules on HL-60 Tyrosine Kinase Activity

A. Agents with no effect:

Compound	Maximum concentration tested
P-Ser	5 mM
P-Thr	5 mM
amiloride	1 mM
levamisole	10 mM
DMSO	20% (v/v)

B. Agents which inhibit activity:

Compound	Concentration (M)	% Activity
None	---	100
P-Tyr	10^{-5}	104
	10^{-4}	102
	10^{-3}	6
Tyrosine	10^{-3}	24
Phenylphosphate	10^{-7}	95
	10^{-6}	92
	10^{-5}	86
	10^{-4}	74
Quercetin	10^{-5}	109
	10^{-4}	64
	10^{-3}	4

Agents were added to the standard tyrosine kinase assay and activity was measured. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

for 10 minutes) completely abolished tyrosine kinase activity (Table 6). The addition of pronase to the particulate fraction (10 minutes at 37°C) also destroyed all activity; this effect was not due to degradation of the substrate, as the addition of pronase after completion of the phosphorylation reaction had no effect on recovery of phosphorylated peptide. The 10 minute incubation at 37°C also was not a significant factor, as a similar incubation with RNase caused no significant diminution in activity.

A number of tyrosine kinases have been shown to have altered activity after the enzymes are pre-incubated with ATP; this modification likely results from the autophosphorylation of the kinase (28-33). To examine this property in the HL-60 preparation, particulate fractions were pre-incubated with ATP for up to 10 minutes. Preincubation for 30 seconds led to a 42% increase in activity; this rose to 64% at 1 minute, and remained at this level for up to 10 minutes thereafter (Figure 19).

The HL-60 tyrosine kinase activity being characterized represents that of unpurified particulate fractions. To try to determine the number of proteins responsible for this activity, basic purification procedures were performed as shown in Table 7. The tyrosine kinase activity was soluble in 25% saturated ammonium sulfate (at 4°C), but was precipitated by 75%. Gel filtration and anion-exchange chromatography each allowed approximately 3-fold

TABLE 6

Stability of HL-60 Tyrosine Kinase to Physical and Enzymatic Treatments

Treatment	% Activity
None	100
Heating (56°C for 10 min)	0
Pronase (250 µg/ml; 10 min at 37°C)	0
RNase A (100 µg/ml; 10 min at 37°C)	97

HL-60 particulate fraction was exposed to the indicated treatment, and tyrosine kinase activity was assessed. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

Figure 19. Effect of pre-incubation with ATP on HL-60 tyrosine kinase activity. Assays were performed as before, except particulate fraction protein was pre-incubated for various times with 25 uM ATP at 23 °C before the assay was performed. Each point represents the mean ± standard error of the mean of two experiments each done in duplicate.

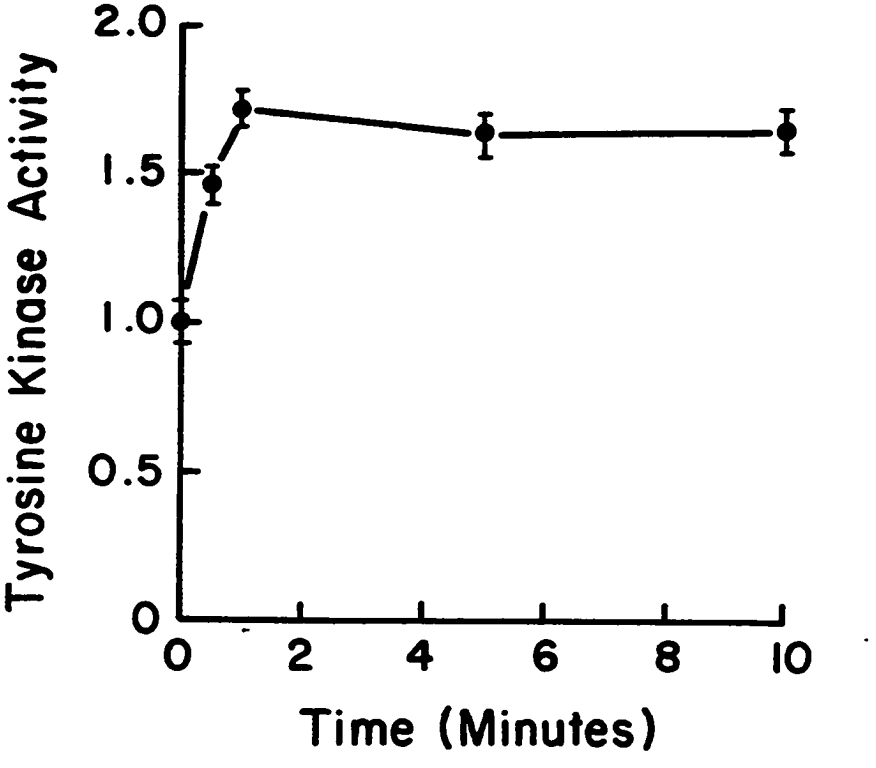


TABLE 7

Purification of HL-60 Tyrosine Kinase Activity

	Protein (mg)	Total activity (pmol/min)	Recovery (%)	Specific activity (pmol/min/mg)	Purification (-fold)
Particulate fraction	54	1296	(=100)	24.0	(=1)
Ammonium sulfate (25-75%)	32	1153	89	36.0	1.5
Sephacryl S-200	8	881	68	108.2	4.5
DEAE-Sephacel	2	583	45	291.4	12.1

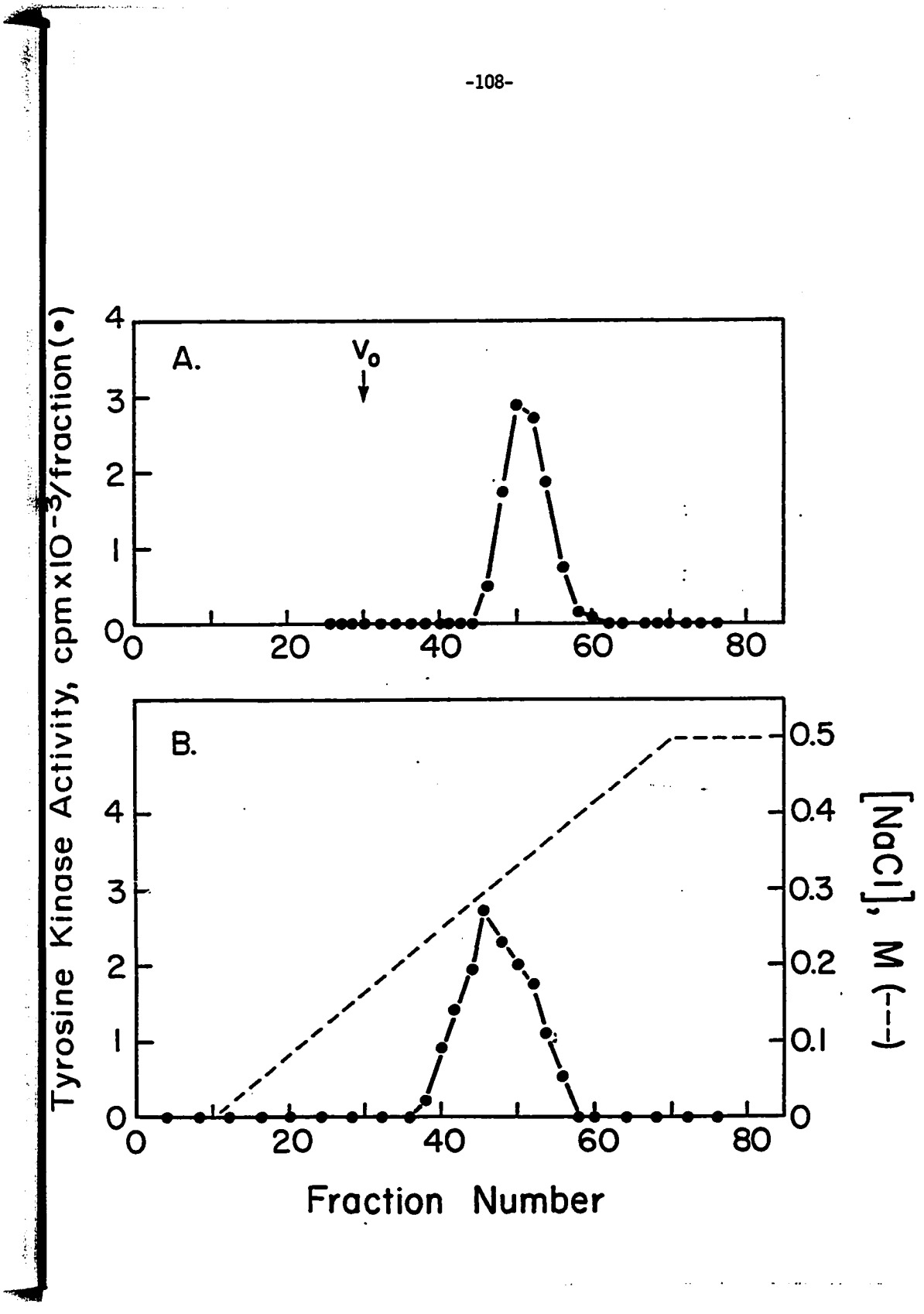
HL-60 particulate fraction tyrosine kinase activity was purified as described in "Materials and Methods," and activity was assessed after each step.

purifications. Although the net purification was low, the eluents from each column showed a single peak of activity (Figure 20).

Characteristics of HL-60 protein phosphotyrosine phosphatase activity. In addition to tyrosine kinase activity, the enzyme activity responsible for regulating intracellular phosphotyrosine levels is the corresponding phosphatase. To measure protein phosphotyrosine phosphatase activity, an appropriate substrate was prepared by exhaustively phosphorylating the tyrosine containing polymer GAT with ^{32}P. As described in "Materials and Methods," particulate fractions from A431 epidermal carcinoma cells (rich in EGF receptors) were used as the kinase in the presence of EGF. In a typical phosphorylation, the kinase and substrate were incubated for 8 hours at 22°C in the presence of a phosphatase inhibitor (25 mM Na_3VO_4). A total of 5.2×10^6 dpm (1.6×10^3 pmol) of phosphate was incorporated into one mg of GAT. As described earlier, one mg of GAT contains about 6×10^{-7} moles of tyrosine, giving a ratio of 2.6×10^{-3} mole phosphate/moles tyrosine (one in 380 tyrosine residues being phosphorylated). Phosphoaminoacid analysis indicated that greater than 95% of the phosphate was in P-tyr.

Phosphatase activity was measured by the release of ^{32}P into a TCA-soluble form. Protease activity, however, could also cleave the substrate into phosphorylated amino

Figure 20. Purification of HL-60 tyrosine kinase activity. Panel A. Gel filtration chromatography on a Sephacryl S-200 column was performed as described in "Materials and Methods." V_o represents void volume. Panel B. Anion-exchange chromatography on a DEAE column was performed on the pooled active fractions from the gel filtration column, as described in "Materials and Methods." Total bed volume was 100 ml (which eluted at fraction 67).



acids and peptides which might be TCA soluble. To exclude this possibility, a molybdate-organic solvent extraction was performed as described in "Materials and Methods." In this extraction, inorganic phospate is partitioned in the organic phase, while phosphopeptides and phosphoaminoacids are recovered in the aqueous phase. Greater than 98% of the released radioactivity was consistently recovered in the organic phase, indicating that the TCA-soluble material was in fact inorganic phosphate.

As with tyrosine kinase activity, greater than 90% of the phosphotyrosine phosphatase activity was found in the particulate fraction. The phosphatase activity was linear with particulate protein concentration to about 25 ug/assay, and it could liberate 100% of the ^{32}P from the substrate (Figure 21). The initial velocity was also linear with substrate concentration to 4 ug/assay, and then plateaued (Figure 22). Activity was temperature dependent, being minimal at $0^{o}C$, and increasing through $37^{o}C$; the reaction was also initially linear with time before reaching a maximum, and in the absence of enzyme, the substrate was extremely stable (Figure 23).

As noted earlier, it has been shown in other systems that the ability to hydrolyze p-nitrophenyl phosphate (PNPPase) may be a property solely of phosphotyrosine phosphatases (136). PNPPase activity in HL-60 cells was almost completely recovered in the particulate fraction

Figure 21. HL-60 phosphotyrosine phosphatase activity as a function of protein concentration. Protein phosphotyrosine phosphatase activity was performed as described in "Materials and Methods" in the presence of varying amounts of particulate fraction protein. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

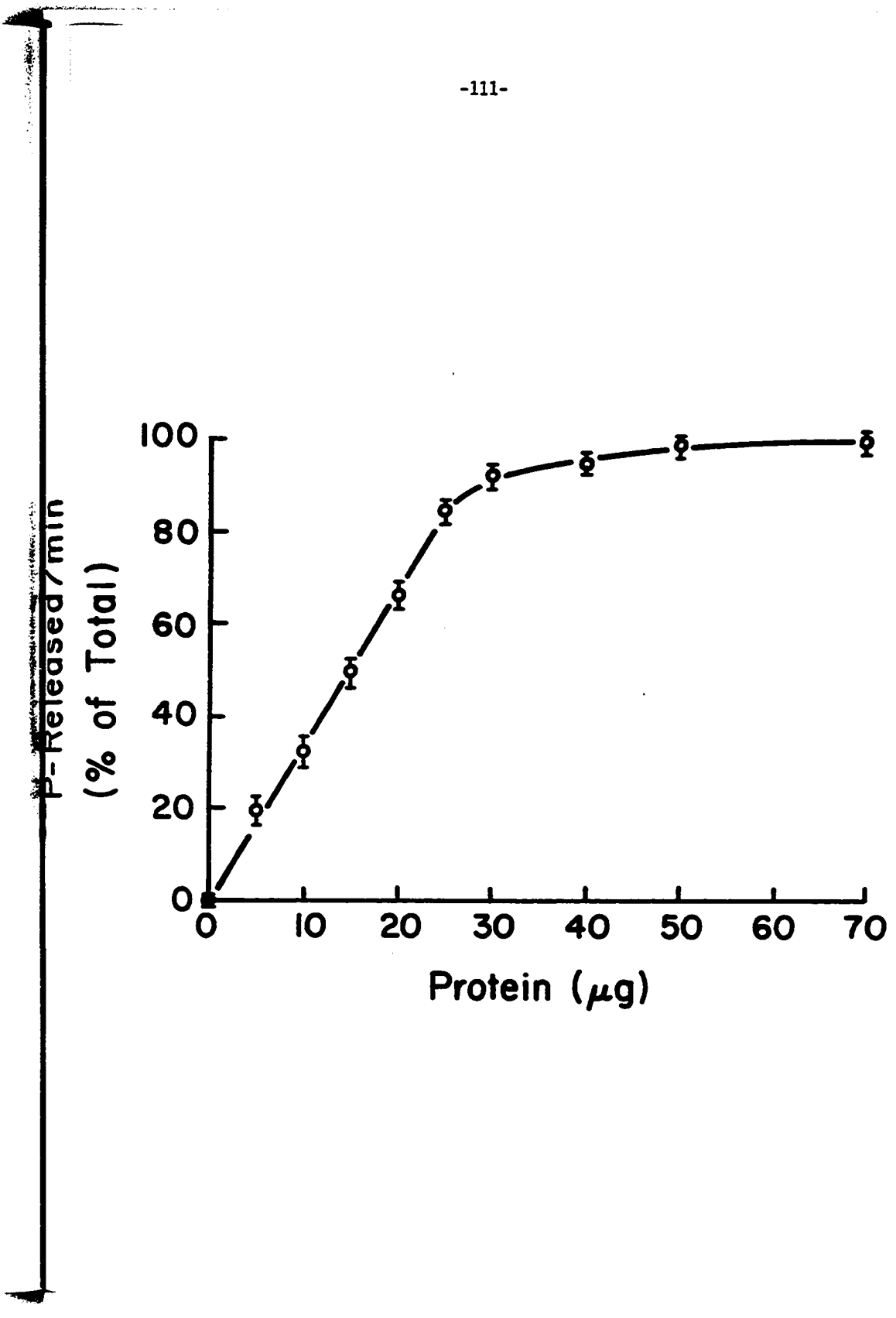


Figure 22. Effect of substrate concentration of phosphotyrosine phosphatase activity. Fifteen micrograms of particulate fraction protein was used for each assay at 22 °C in the presence of varying concentrations of substrate. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate

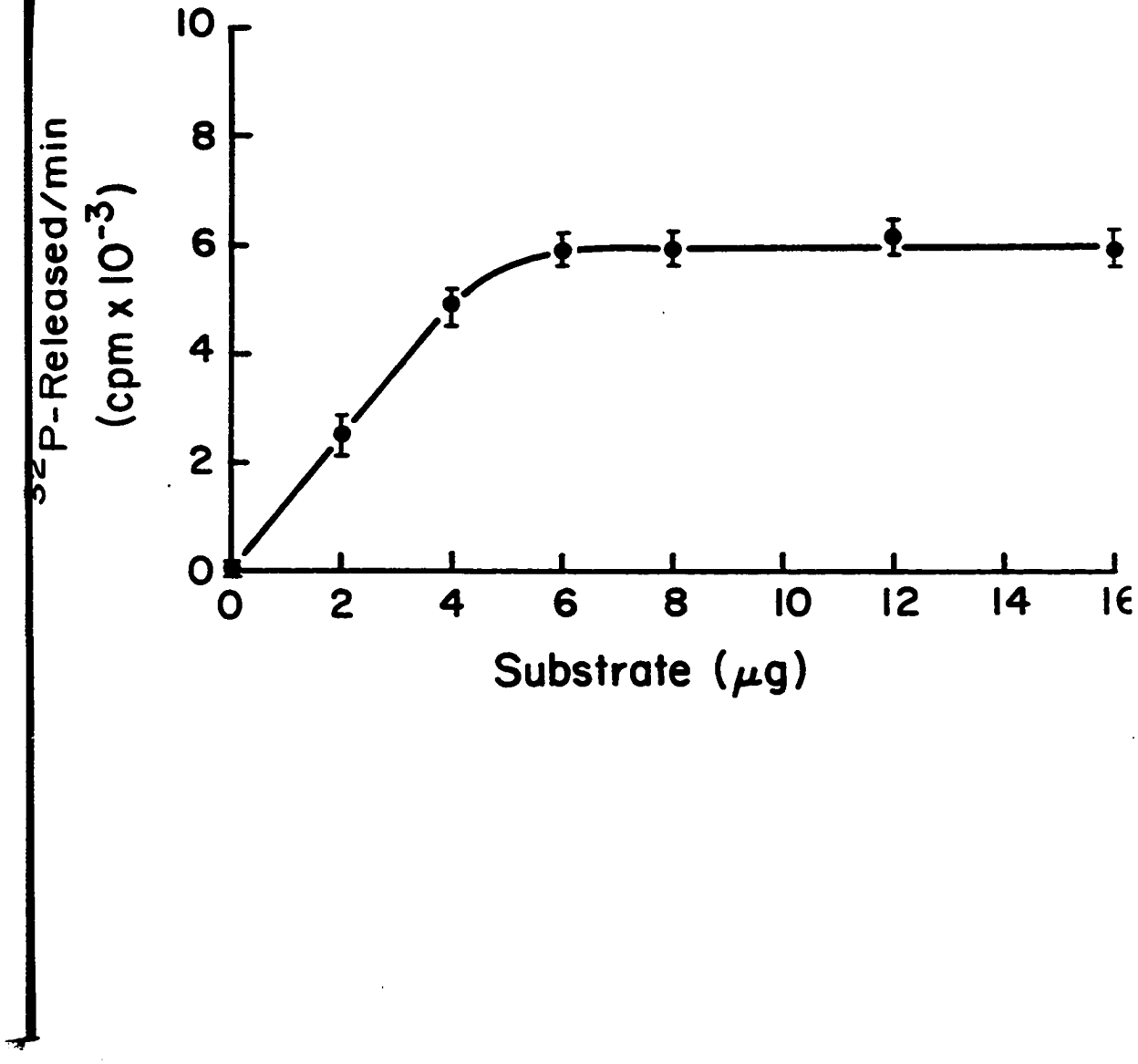
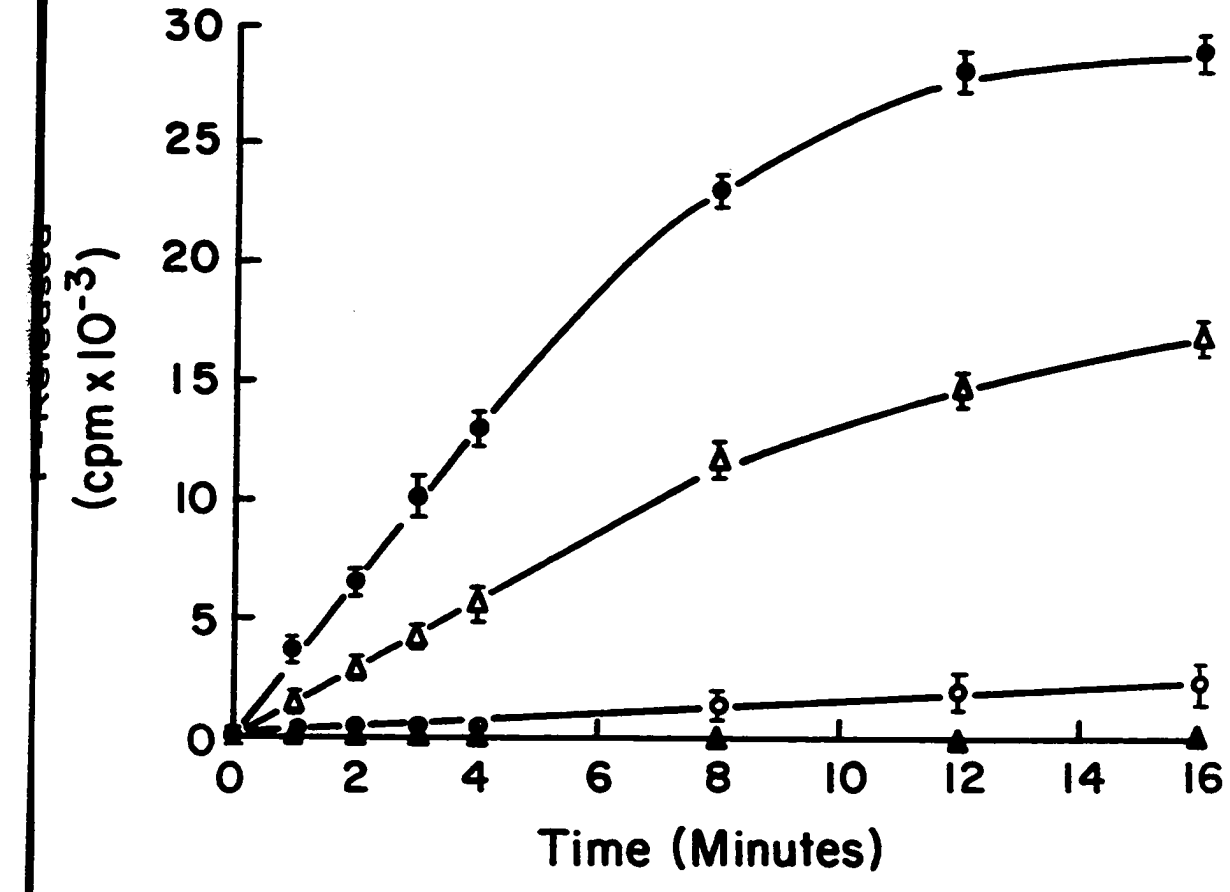


Figure 23. Temperature dependence of phosphotyrosine phosphatase. Phosphatase assays were performed using 10 ug of particulate fraction protein at 0 °C (○), 22 °C (△), 37 °C (●), and in the absence of protein (▲). Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.



(greater than 90%). As with the phosphatase, PNPPase activity was linear with protein concentration (Figure 24); the K_m for PNPP was approximately 1.9 mM (Figure 25). PNPPase activity also showed a similar time course and temperature dependence as the phosphatase (Figure 26).

The responses of protein phosphotyrosine phosphatase and PNPPase to a number of ions and small molecules closely paralleled each other. Neither EDTA (up to 0.5 mM), $MnCl_2$ (up to 1 mM), nor $MgCl_2$ (up to 1 mM) had a significant effect on either activity (Table 8). $ZnCl_2$, however, caused nearly complete inhibition at 10 mM.

Much work has been done on the similarities between tyrosine kinases and phosphatidyl inositol (PI) kinases, and it has been suggested that some of the effects of the classically described tyrosine kinases are actually mediated through PI phosphorylations (55). Li^+ is a potent inhibitor of inositol phosphate phosphatases (156), and it may exert its clinical effects through a mechanism in which inositol tris-phosphate builds up because the phosphate groups cannot be cleaved. It had been noted that patients being treated with Li^+ for manic-depressive illness often exhibit a leukocytosis, and it was determined that Li^+ leads to an increased production of leukocytes by the bone marrow (57). It had been suggested that Li^+ might be a powerful adjunct in anti-leukemic treatment, by helping to repopulate the

Figure 24. Effect of protein concentration on PNPPase activity. Assays were performed as described in "Materials and Methods," using various particulate fraction protein concentrations. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

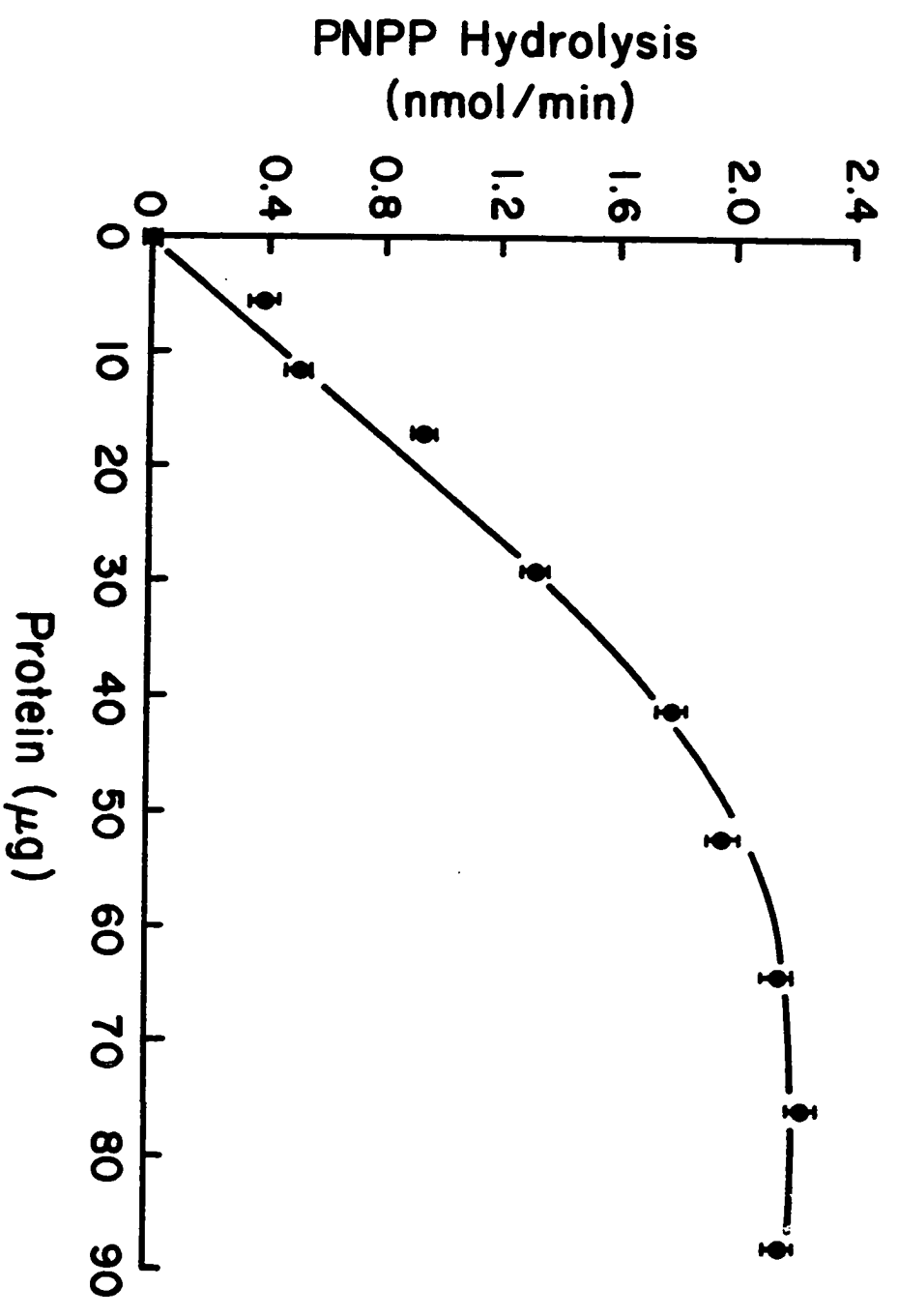


Figure 25. Effect of substrate concentration on PNPPase activity. Assays were performed as described in the presence of varying concentrations of substrate. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate. Inset: Data plotted in double reciprocal fashion.

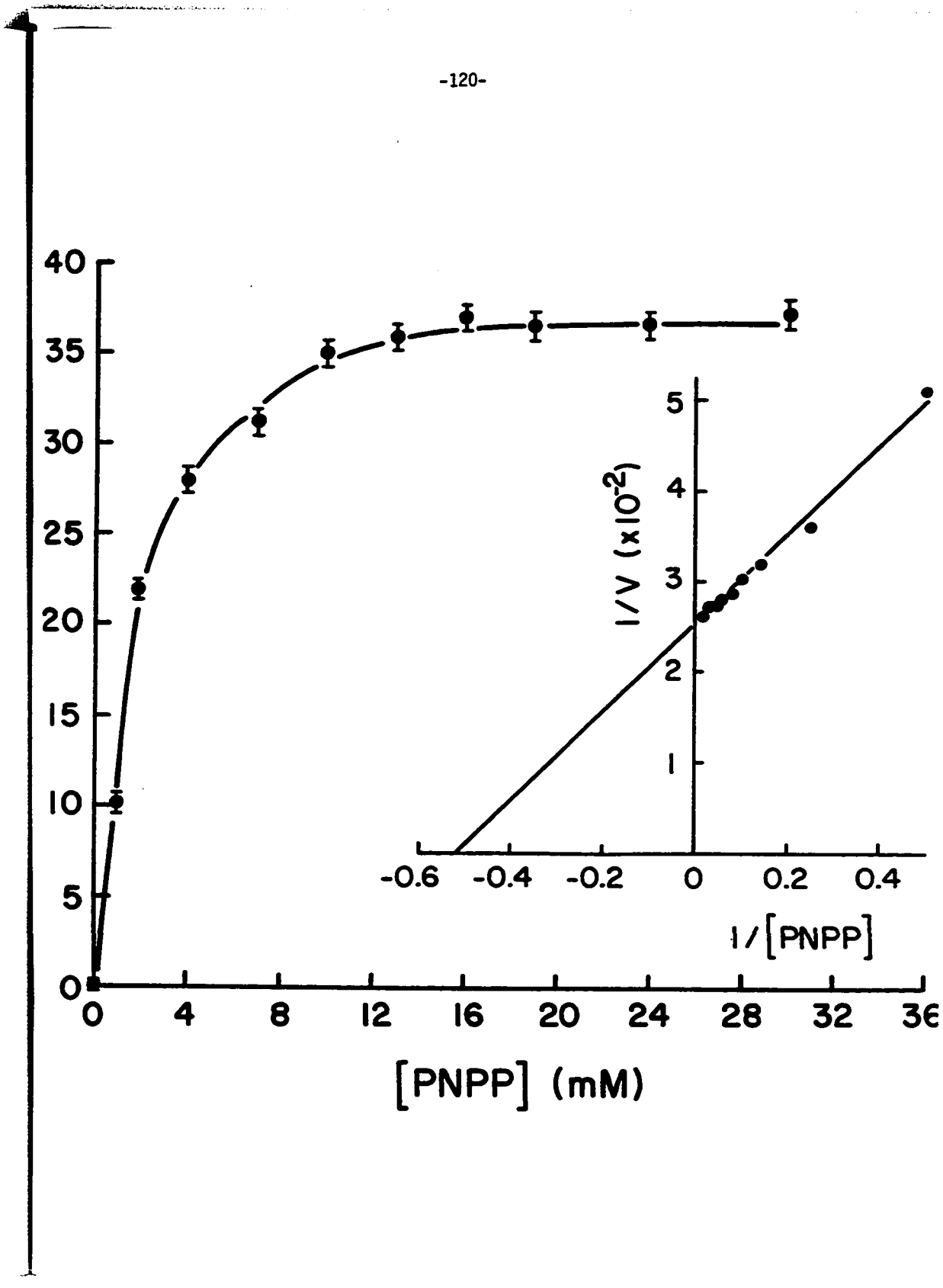


Figure 26. Temperature dependence of PNPPase activity. Assays were performed at 0 °C (○), 23 °C (△), 37 °C (●), and in the absence of protein (▲). Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

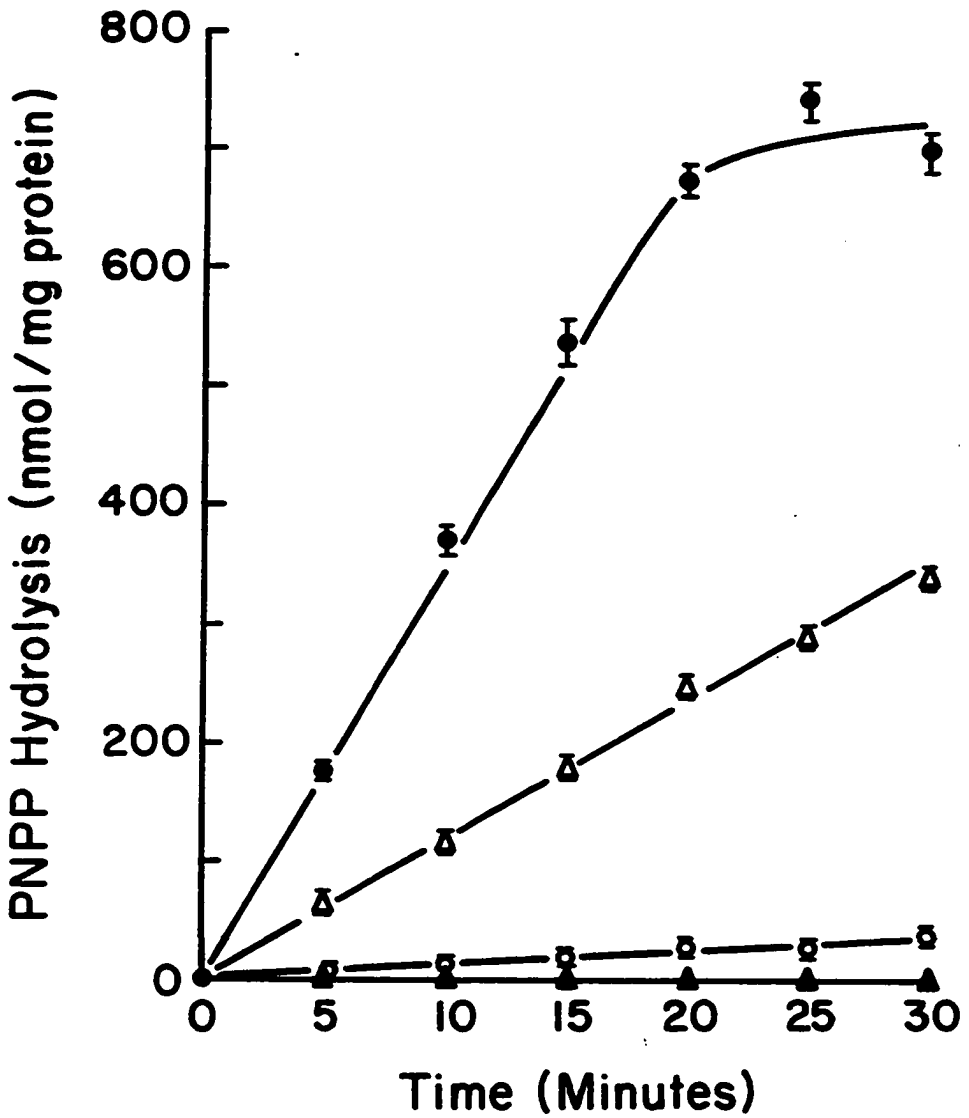


TABLE 8

Effect of Various Compounds on HL-60 Phosphotyrosine Phosphatase and PNPPase Activities

A. Agents which inhibit activity:

Compound	Concentration	% Activity PNPPase	% Activity Phosphatase
None	—	100	100
$ZnCl_2$	10^{-6}M	98	102
	10^{-5}	96	94
	10^{-4}	84	82
	10^{-3}	27	19
	10^{-2}	6	6
NaF	5×10^{-5}M	97	96
	5×10^{-4}	89	77
	5×10^{-3}	69	58
	5×10^{-2}	24	35
Na_3VO_4	10^{-7}M	97	102
	10^{-6}	87	79
	10^{-5}	35	28
	10^{-4}	18	11
	10^{-3}	1	7
	10^{-2}	0	0
NaCl	5×10^{-2}M	100	96
	5×10^{-1}	46	54
LiCl	10^{-3}M	98	102
	10^{-2}	85	91
SDS	0.001% (w/v)	95	97
	0.01	38	28
	0.1	0	0

B. Agents which have no effect:

Compound	Maximum concentration tested
ATP	1 mM
inorganic pyrophosphate	10 mM
quercetin	10^{-4} M
levamisole	10^{-2} M

Compounds were added to standard assays as described in "Materials and Methods," and activities were assessed. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

bone marrow following the hypoplastic or aplastic phase caused by induction chemotherapy. It was subsequently discovered, however, that Li^+ led to an increase in the proliferative capacity of several leukemic cell lines, including HL-60 (131). Although the mechanism of this effect has not been elucidated, it is conceivable that the effects of Li^+ are being mediated through an inhibition of phosphotyrosine phosphatase. To examine this possibility, the effects of LiCl on PNPPase and phosphotyrosine phosphatase activity were explored. A small (10-15%) inhibitory effect was seen at 10 mM (therapeutic serum levels are 0.5 to 1.5 mM); ionic strength per se was not a factor as NaCl showed no effect until a concentration of 500 mM (Table 8).

A known inhibitor of many serine/threonine phosphatases is NaF. One of the first described distinctive features of phosphotyrosine phosphatases was the unusual response to the fluoride ion. The enzyme described by Nelson and Branton (158) and the phosphotyrosine phosphatase activity of the alkaline phosphatases described by Swarup et al. (77) were both resistant to inhibition by F^-. The activity examined in A431 membranes by Brautigan et al. (75) was actually stimulated slightly by fluoride, an effect the authors suggested was due to the complexing and removal of Zn^{2+} by this anion. The HL-60 phosphotyrosine phosphatese was inhibited somewhat by fluoride, with 65% inhibition found at 50 mM (Table 8). This feature apparently

distinguishes it from other such enzymes described. The most potent inhibitor of the phosphatase and PNPPase was orthovanadate, which at 1 mM inhibited greater than 90% of both activities (Table 8).

The ionic detergent SDS caused 60 to 70% inhibition at a concentration of 0.001% (w/v) and complete inhibition at higher concentrations. Neither quercetin (to 10^{-4} M), levamisole (to 10^{-2} M), ATP (to 1 mM), nor inorganic pyrophosphate (10 mM) altered these activities. The addition of 10 mM PNPP to the phosphatase assay decreased activity by 14% (Table 8).

The pH optima varies slightly between the two activities, being pH 8 for the phosphatase and pH 7 for the PNPPase (Figure 27). The phosphatase has activity as low as pH 4 and as high as pH 9, while the range for PNPPase is 3 to 8. To determine whether commercially available alkaline or acid phosphatases possess phosphotyrosine phosphatase activity, three such proteins were tested in this assay. Two alkaline phosphatases, one from pig kidney and one from calf intestine, hydroylysed PNPP in a pH range from 9 to 14 (maximal at 11), but failed to cleave P-tyr at any pH (Figure 28). Prostatic acid phosphatase possessed PNPPase activity with a maximum at pH 4 and a range of 3 to 8. The acid phosphatase did cleave the P-tyr containing substrate slightly at pH 5 to 7, though the specific activity of the commercial preparation was less than one-tenth that of the HL-60 phosphatase (Figure 29).

Figure 27. pH dependence of phosphotyrosine phosphatase and PNPPase activity. Each assay was performed at the indicated pH in Hepes buffer. Each point represents the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

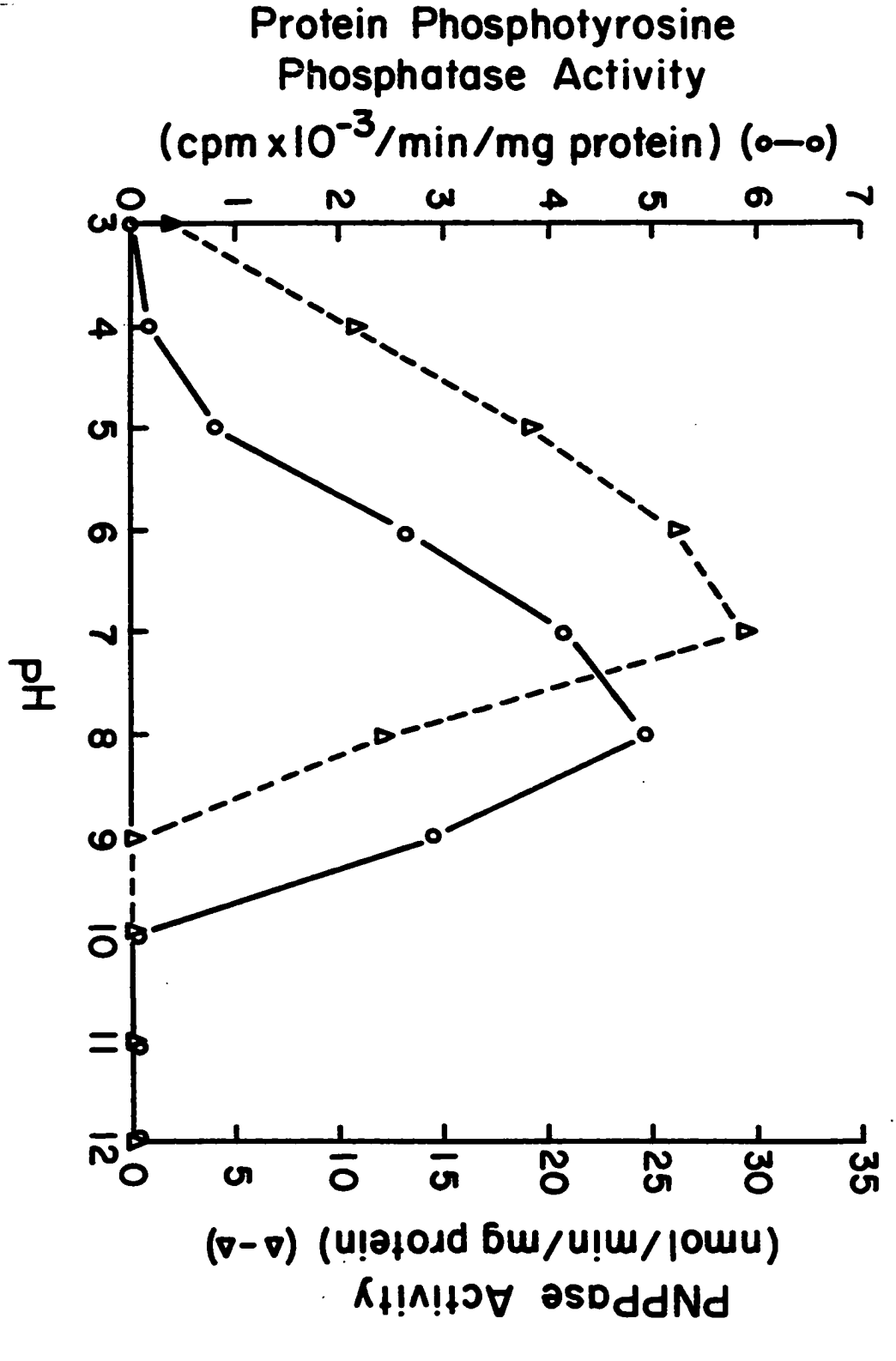


Figure 28. Activity of commercially available alkaline phosphatases on PNPP and phosphotyrosine hydrolysis. Pig kidney (filled symbols) and calf intestine (open symbols) alkaline phosphatases (from Sigma, St. Louis, Mo, and Miles Laboratories, West Haven, CT, respectively) were assayed for PNPPase (triangles) and phosphotoyrosine phosphatase (circles) activities. Each point represents the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

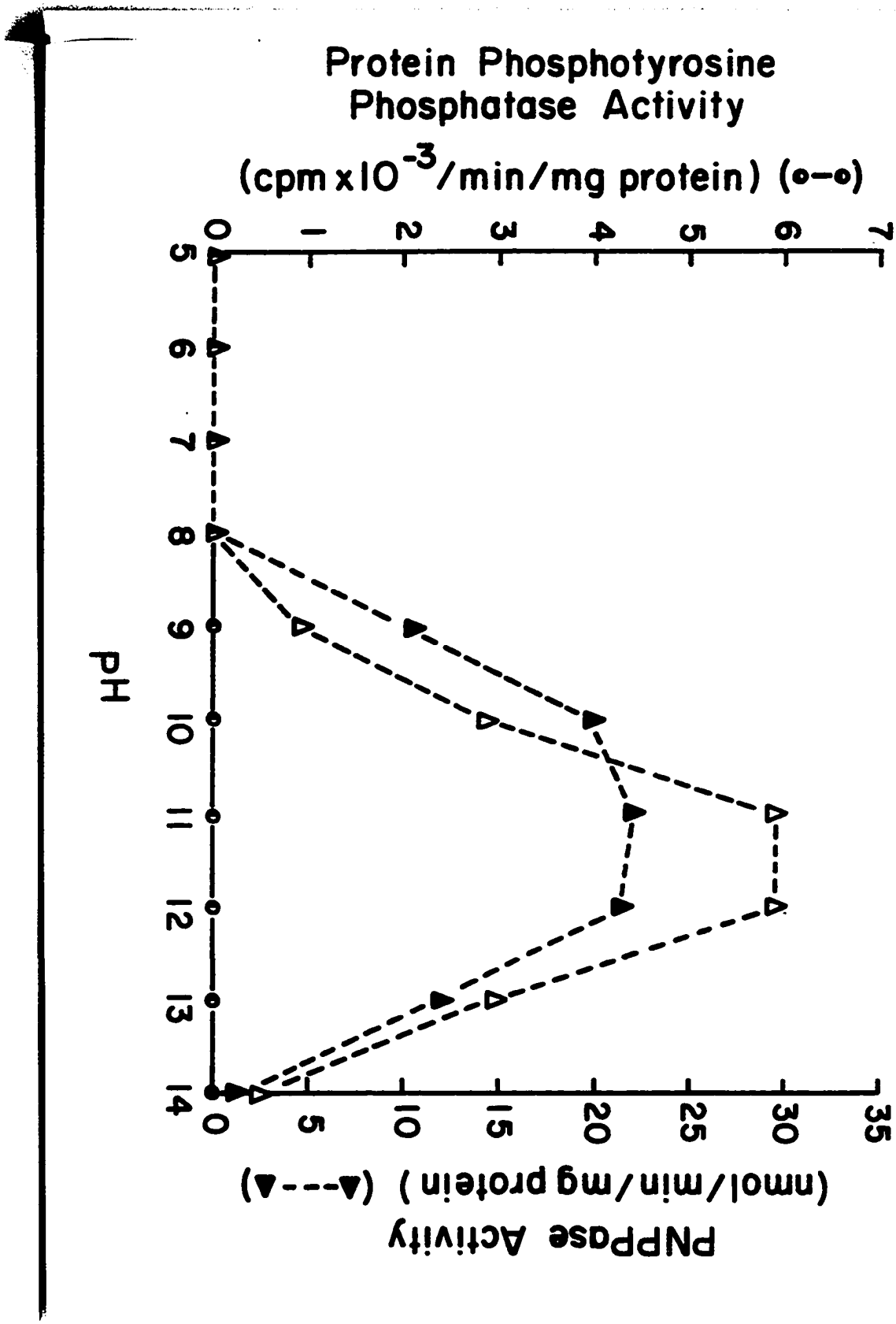
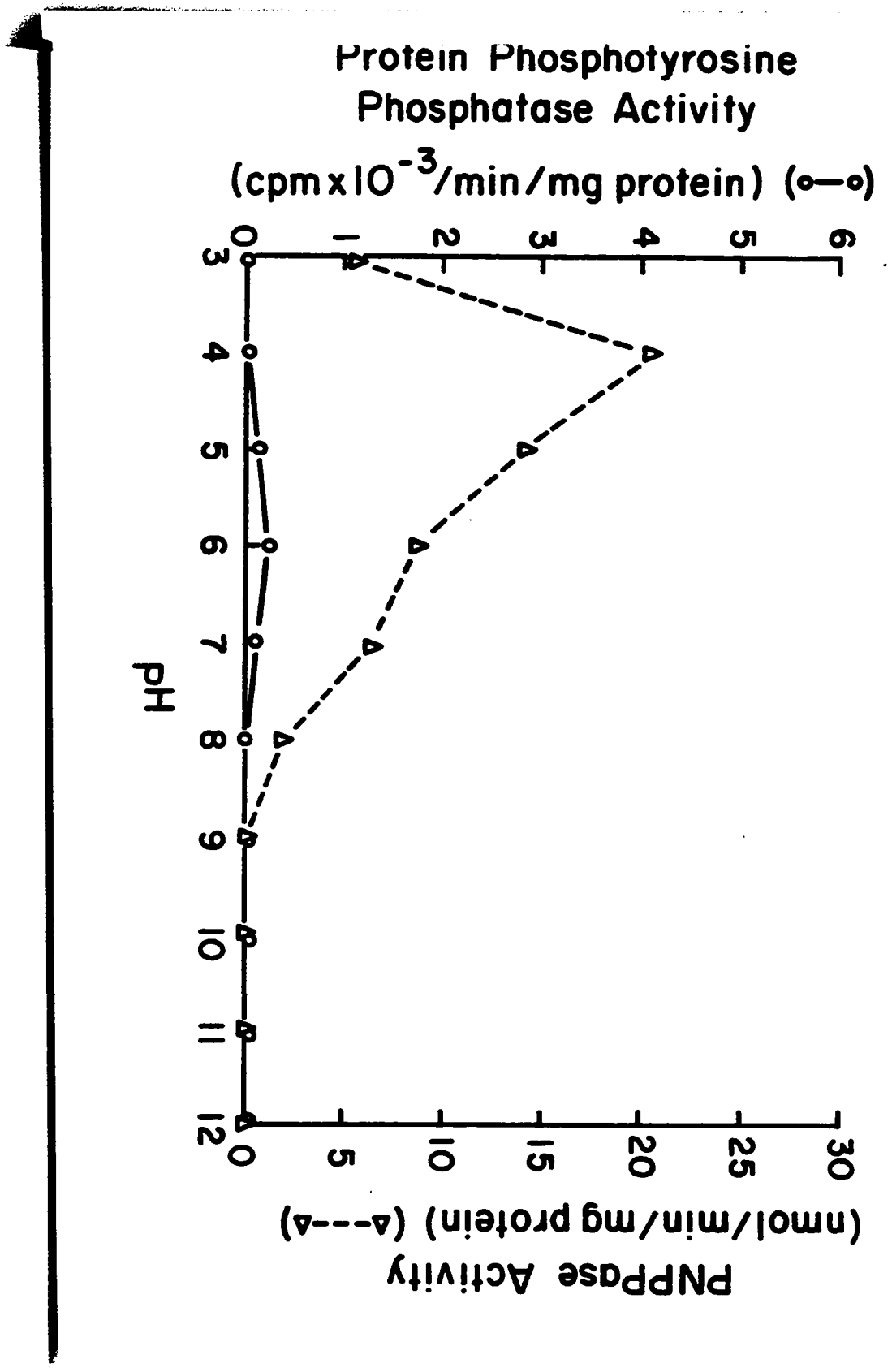


Figure 29. Activity of commercially available acid phosphatase on PNPP and phosphotyrosine hydrolysis. Prostatic acid phosphatase (Sigma) was assayed for PNPPase and phosphotyrosine phosphatase activities. Each point represents the mean of two experiments each done in duplicate; the range was less than 5% of the mean.



As with the kinase, the question arose as to the number of proteins in the particulate fraction that were responsible for the phosphatase activity. Before attempting a purification, the thermal stability of the phosphatase activity was assessed. A one hour incubation at 60°C abolished all activity; 50% activity was lost after 8 hours at 37°C, 15 hours at 22°C, and 60 hours at 4°C (Figure 30).

A purification scheme identical to that of the tyrosine kinase was employed for the phosphotyrosine phosphatase (Table 9). As with the kinase, the phosphatase was recovered in the 25% to 75% ammonium sulfate soluble fraction. This step followed by gel filtration and anion exchange chromatography allowed a net 14-fold purification. Of note, however, was that the activity recovered from each of these columns eluted as a single peak (Figure 31).

Subcellular localization of HL-60 tyrosine kinase and phosphotyrosine phosphatase activities. As the particulate fraction contains all cellular membranes, an effort was made to further define the location of this activity. Employing the technique of Hertel et al. (130) described in the "Materials and Methods," plasma membranes were separated from internal cellular membranes by density isolation. This method relies on the ability of concanavalin A to cross-link surface membrane proteins such that when cells are lysed by hypotonic treatment, the plasma membrane forms sheets while the internal membranes remain as vesicles. A step sucrose

Figure 30. Thermal stability of phosphotyrosine phosphatase activity. Particulate fraction protein was incubated at 4°C (○), 23°C (▲), 37°C (●), and 60°C (△), and phosphotyrosine phosphatase activity was measured at varying intervals.

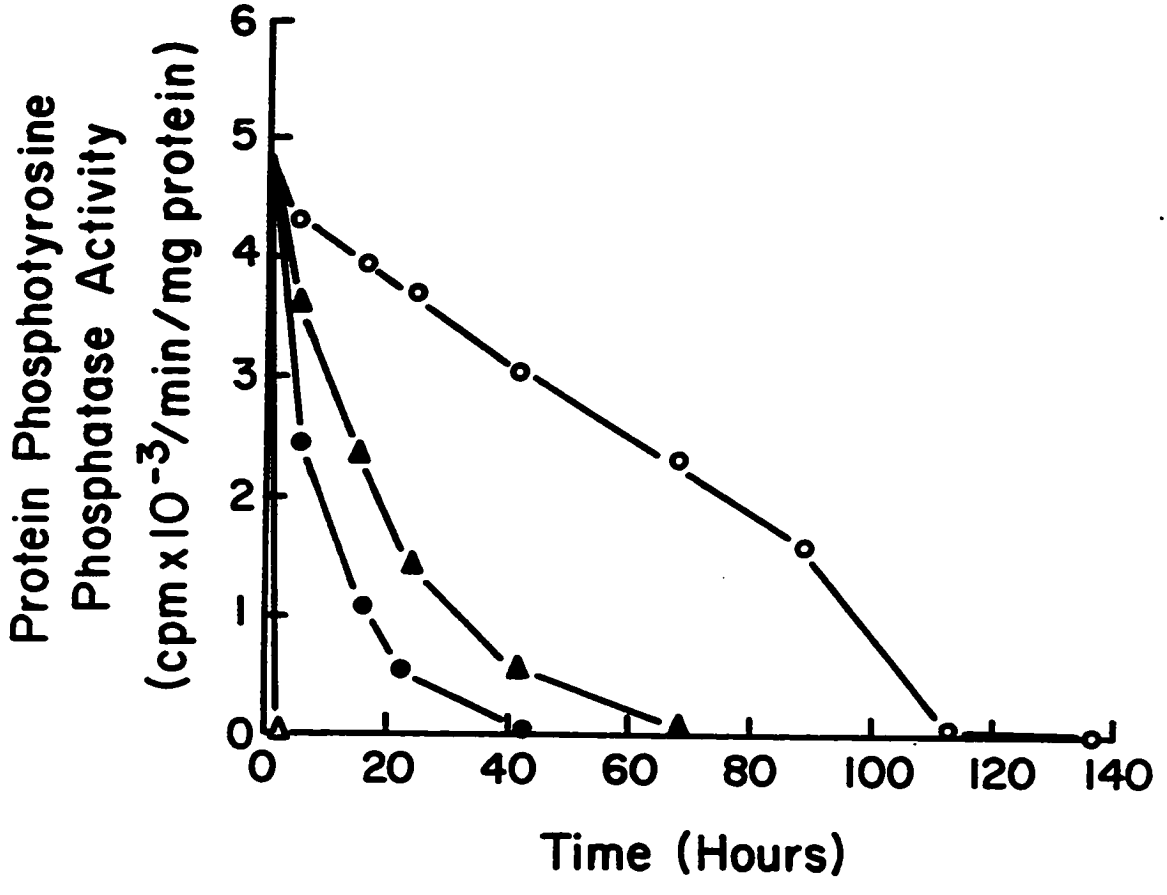


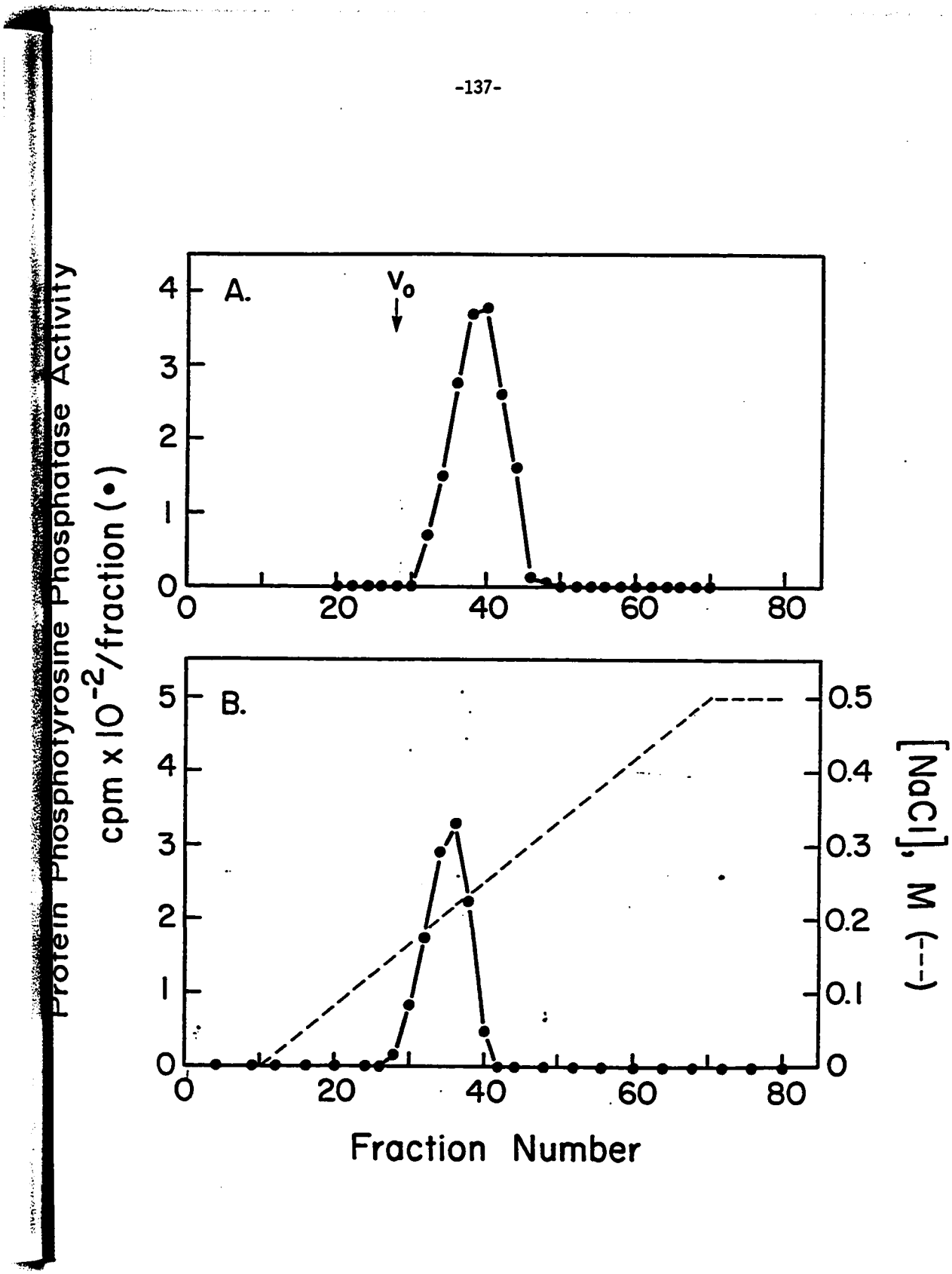
TABLE 9

Purification of HL-60 Protein Phosphotyrosine Phosphatase Activity

	Protein (mg)	Total activity (cpm x 10^2/min)	Recovery (%)	Specific activity (cpm x 10^2/min/mg)	Purification (-fold)
Particulate fraction	61	2987	(=100)	49.0	(=1)
Ammonium sulfate (25-75%)	40	2778	93	69.5	1.4
Sephacryl S-200	9	2084	75	231.5	4.7
DEAE-Sephacel	3	2001	67	667.1	13.6

HL-60 particulate fraction phosphotyrosine phosphatase activity was purified as described in "Materials and Methods," and activity was assessed after each step.

Figure 31. Purification of phosphotyrosine phosphatase activity. A. Particulate fraction extracts were subjected to gel filtration on a Sephacryl S 200 column, and the activity of the eluate assayed. V_o represents void volume. B. The pooled active fractions from the gel filtration were subjected to DEAE anion-exchange chromatagraphy, as described in "Materials and Methods." Total bed volume was 100 ml, which eluted at fraction 67.



gradient can then be employed to obtain quantitative separation and recovery of each fraction (130). Using this technique, it was found that five times as much tyrosine kinase resides on the plasma membrane as on internal membranes; conversely, nearly four times as much of the phosphatase activity is located on the internal membranes (Table 10A).

Additional experiments were directed at further defining the subcellular location of these enzymes. Given that they are predominantly membrane-associated, the question arose as to which face of the membrane they resided upon. The technique of Chaney and Jacobson (129) was employed to recover pure plasma membranes with only the cytoplasmic face available for enzymatic reactions. This method (described in "Materials and Methods") relies on the formation of a thick pellicle around the external face of the plasma membrane. Cationic silica is used to coat the outside membrane with a positively charged layer; this is then followed by treatment with polyacrylic acid to form an anionic layer. This technique is then repeated to form a thick pellicle. Lysis of the cells (by freeze/thaw cycles) and centrifugation then allows recovery of plasma membranes with only the cytoplasmic phase exposed. Cells were exposed to trypsin (0.1% (w/v) for 5 minutes at 37°C) either before pellicle formation or after the plasma membranes were recovered. As seen in Table 10B, trypsinization after

TABLE 10

A. **Subcellular Distribution of Tyrosine Kinase and Phosphotyrosine Phosphatase Activities**

	Relative Specific Activity	
	plasma membrane	internal membrane
Tyrosine kinase	0.84	0.16
Phosphotyrosine phosphatase	0.22	0.78

Cellular membranes were separated as described in "Materials and Methods," and enzyme activities were determined. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

B. **Plasma Membrane Orientation of Tyrosine Kinase and Phosphotyrosine Phosphatase Activities**

Trypsinization before pellicle formation	Trypsinization after isolation	Tyrosine kinase activity (pmol x 10^2/min)	Phosphotyrosine phosphatase activity (cpm x 10^2/min)
---	---	37.1	23.4
---	+	0.9	0.0
+	---	34.9	22.2
+	+	0.0	0.0

Plasma membranes were isolated as described in the text. Membranes were exposed to trypsin (0.05% at 37°C for 10 min) either before pellicle formation (to remove outwardly exposed proteins) or after isolation (to remove inwardly exposed proteins) and enzyme activities were assessed. Values represent the mean of two experiments each done in duplicate; the range was less than 5% of the mean.

membrane isolation completely abolished both kinase and phosphatase activities. This indicates that at least some of these activities resides on the cytoplasmic face of the membrane.

Changes in HL-60 tyrosine kinase and protein phosphotyrosine phosphatase activities with granulocytic differentiation. As demonstrated in Figure 1, HL-60 cells treated with DMSO (1.2%; v/v) or RA (10^{-6} M) differentiate into mature granulocytes. During this maturation, cellular phosphotyrosine content decreases by about 10-fold, as measured as a proportion of phosphoaminoacids (Table 1). The next series of experiments was designed to examine how the enzyme activities which govern cellular P-tyr content varied with granulocytic differentiation. HL-60 cells treated with DMSO or RA exhibit a 3- to 5-fold increase in tyrosine kinase activity after 6 to 7 days of drug exposure (Figure 32). This increase begins slightly before an increase in functionally mature cells becomes evident. As untreated cells enter the plateau phase, they show a small increase in tyrosine kinase activity. Protein phosphotyrosine phosphatase activity also increased with differentiation, from 6- to 8-fold; as with the kinase, the increased began slightly before the mature phenotype became evident (Figure 33). Untreated cells exhibited a small increase in activity as they entered plateau phase. Qualitatively and quantitatively similar results were observed when PNPPase activity was measured (Figure 34).

Figure 32. Tyrosine kinases activity in HL-60 cells treated with DMSO and RA. Cells were treated with 1.2% (v/v) DMSO (●), 10^{-6} M RA (▲), or untreated (○), and tyrosine kinase activity from the particulate fractions were assayed at the indicated times. Each point represents the mean ± standard error of the mean of five experiments each done in duplicate.

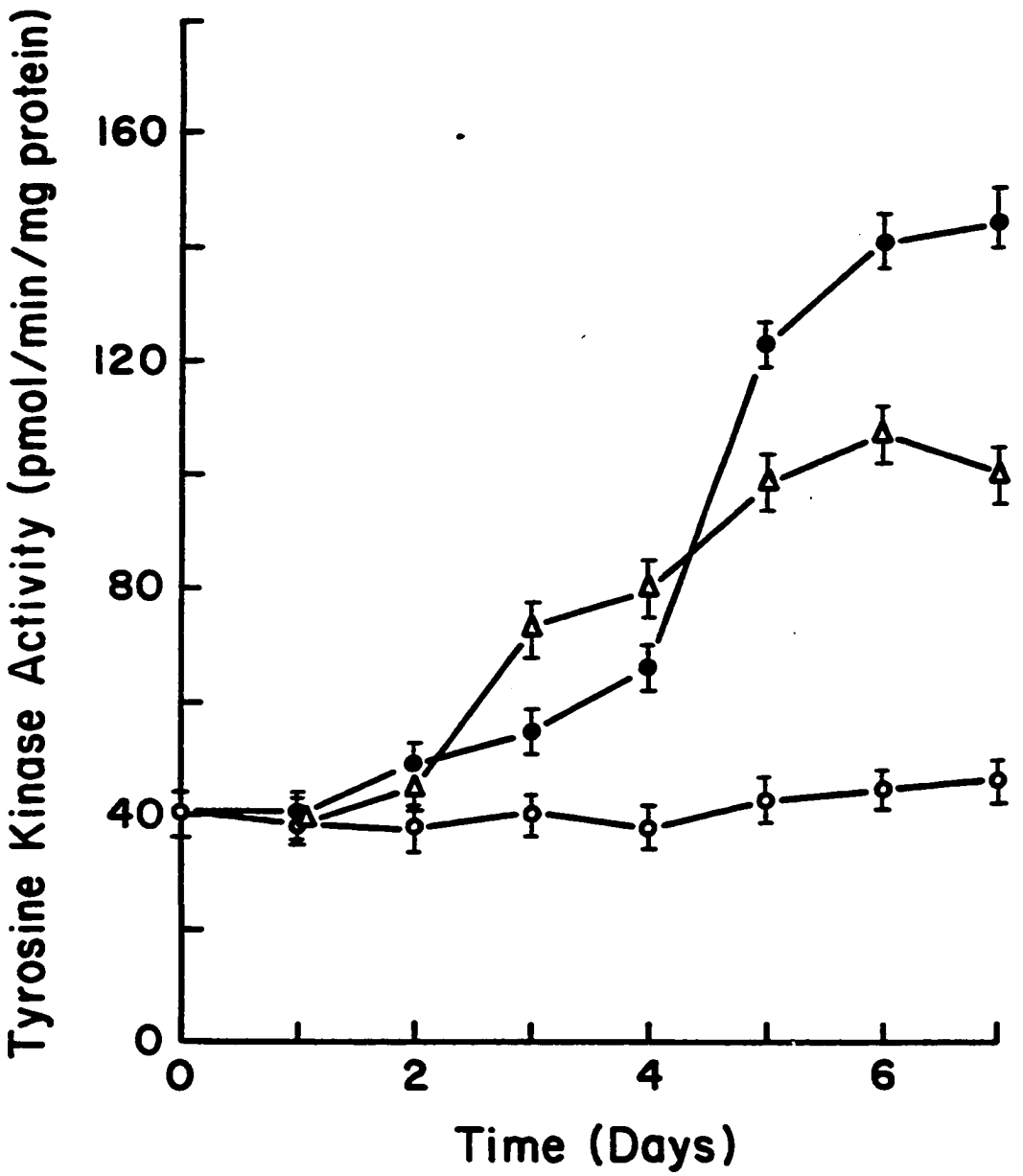


Figure 33. Phosphotyrosine phosphatase activity of HL-60 cells treated with DMSO and RA. Cells were treated with 1.2% (v/v) DMSO (●), 10^{-6} M RA (△), or untreated (○), and phosphotyrosine phosphatase activity was assayed at the indicated times. Each point represents the mean ± standard error of the mean of five experiments each done in duplicate.

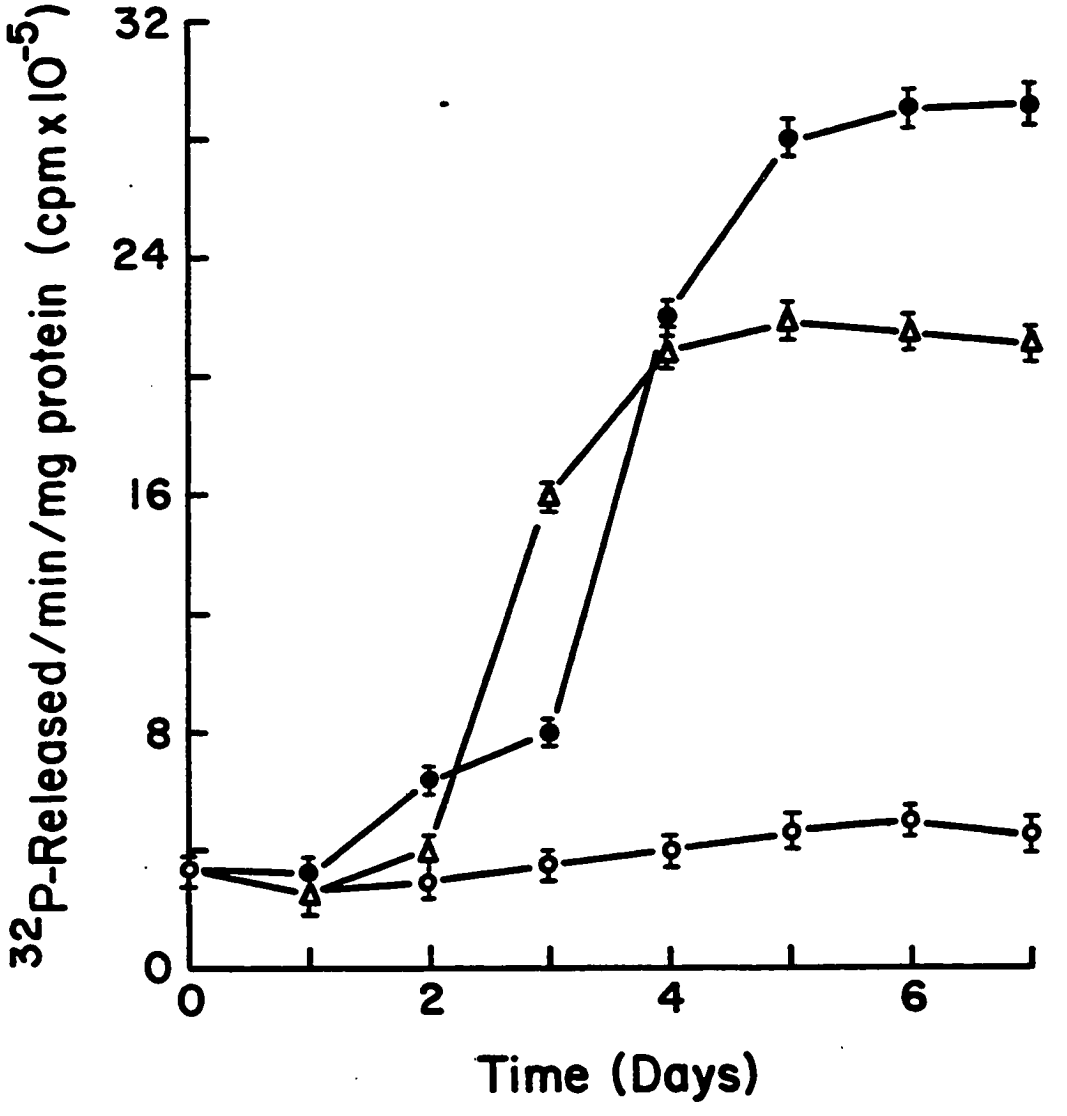
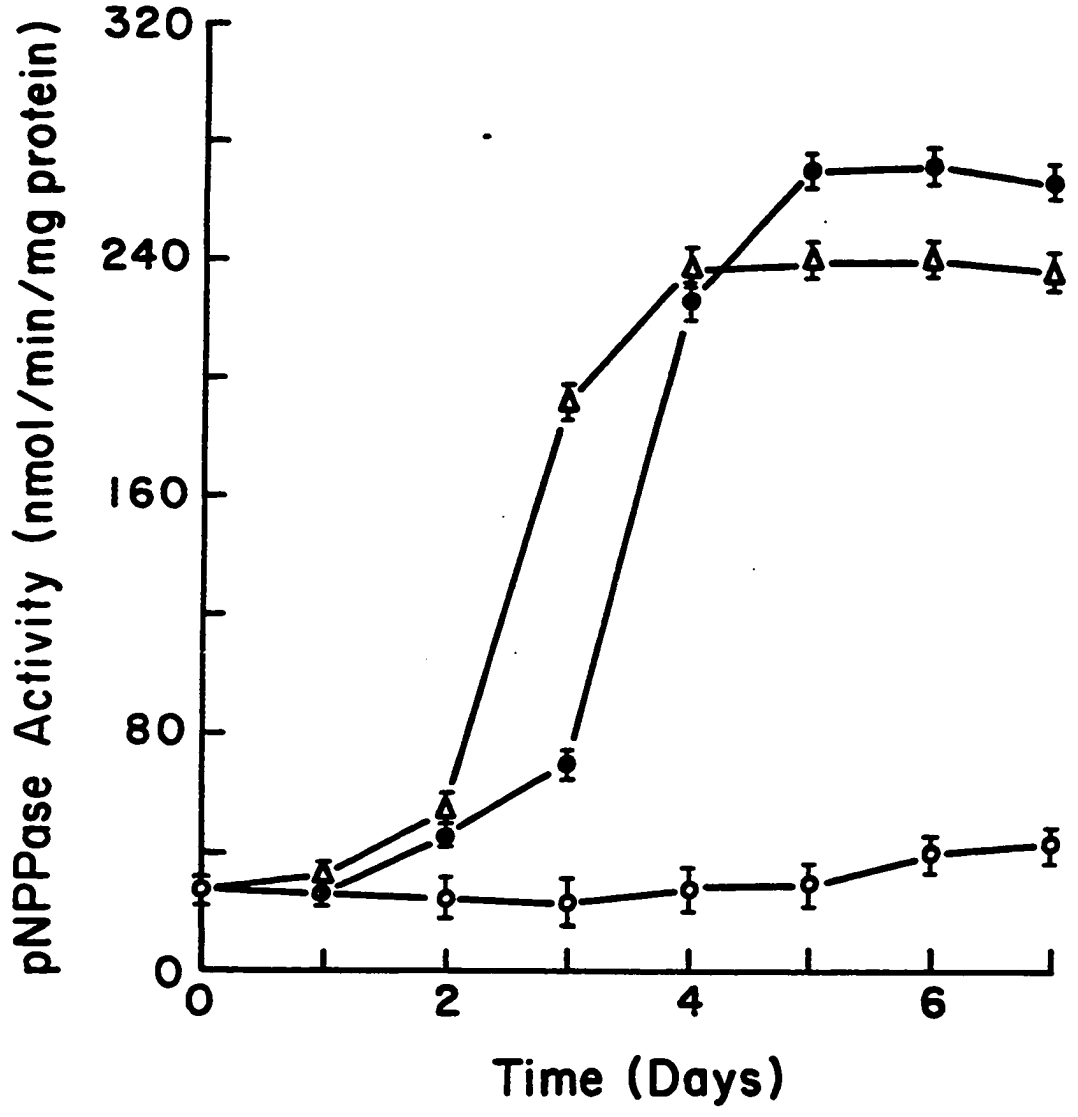


Figure 34. PNPPase activity of HL-60 cells treated with DMSO and RA. Cells were treated with 1.2% (v/v) DMSO (●), 10^{-6} M RA (△), or untreated (○), and PNPPase activity was assayed at the indicated times. Each point represents the mean ± standard error of the mean of five experiments each done in duplicate.



The finding of similar changes in the DMSO- and RA-treated cells strongly suggested that the alterations observed in these enzyme activities were specific for the differentiated cells. As the tyrosine kinase is sensitive to the presence of detergents (Table 4), and as DMSO may interact with cellular membranes, the effect of this agent directly on the kinase was examined. One tyrosine kinase is known to exhibit a doubling of activity in 10% (v/v) DMSO (169). In concentrations up to 20% (v/v), DMSO had no effect on this activity in HL-60 particulate fraction (Table 5). To further exclude a non-specific effect by DMSO, two HL-60 sublines (HL-60/DMSO Y1 and HL-60/DMSO Y2) resistant to DMSO-induced differentiation, were derived by selection in DMSO-containing medium (as described in "Materials and Methods"). Both sublines had the property that they were dependent on DMSO (1.2% v/v) for growth, yet they would not differentiate in its presence (Figure 35). Upon the addition of 10^{-6} M RA, however, the cells did differentiate to a similar extent as the parent line, indicating that they had not lost the ability to manifest the mature state. Both sublines grew with a doubling time of 37 hours (compared to 28 hours for the parent); the plateau density of 2.5×10^{6} cells/ml was nearly identical to that of the parent cells (Figure 36). The addition of 10^{-6} M RA caused the cells to cease proliferating after 3 to 4 days at a density of about 4.5×10^{5} cells/ml.

Figure 35. Differentiation of HL-60/DMSO Y1 and Y2 cells treated with DMSO and RA. HL-60/DMSO Y1 (circles) and Y2 (triangles) were either untreated (open) or treated (filled) with 10^{-6} M RA, and the ability to reduce nitroblue tetrazolium assessed at the indicated times. All cultures contained 1.2% (v/v) DMSO, which was necessary for propagation of the cells. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

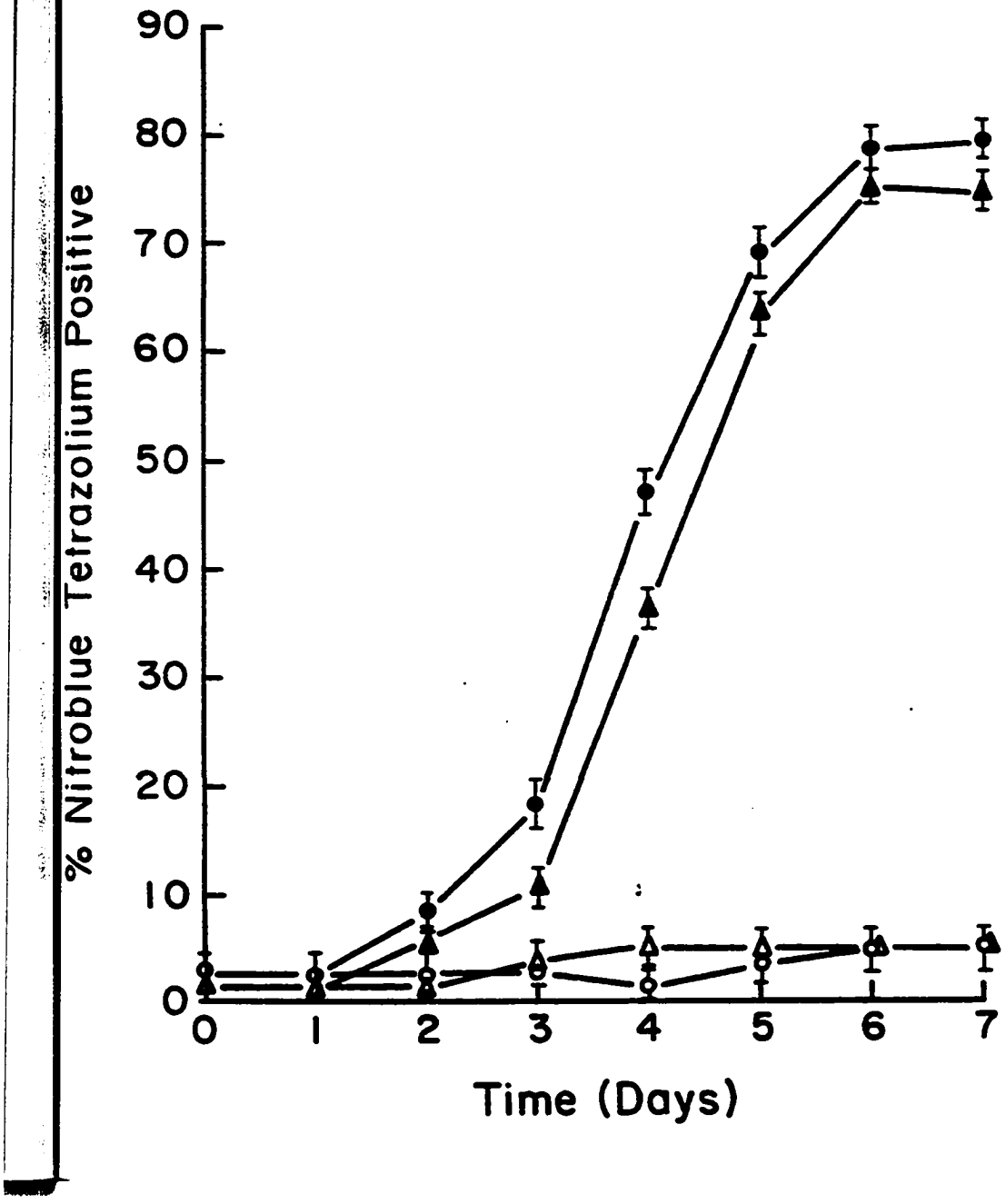
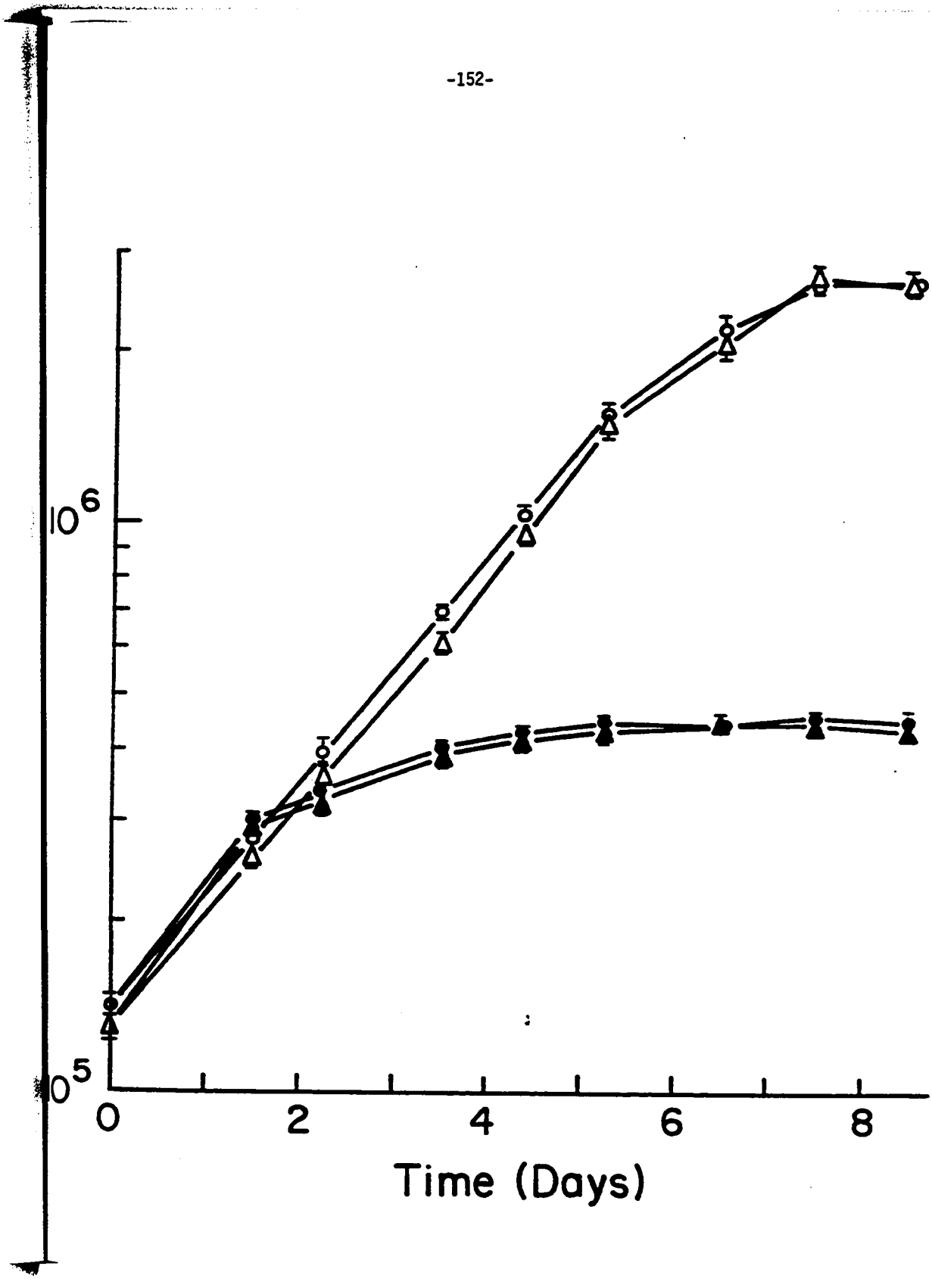


Figure 36. Growth of HL-60/DMSO Y1 and Y2 cells treated with DMSO and RA. HL-60/DMSO Y1 (circles) and Y2 (triangles) were either untreated (open) or treated (filled) with 10^{-6} M RA, and cell number determined as described. Viability was consistently greater than 90%. All cultures contained 1.2% (v/v) DMSO to maintain cell propagation. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.



These sublines have a baseline P-tyr content somewhat lower than the parent cells (Table 11), though following RA-induced differentiation, these levels are similar to those of mature HL-60 cells. Conversely, the tyrosine kinase (Figure 37), protein phosphotyrosine phosphatase (Figure 38A), and PNPPase (Figure 38B) activities are all 10 to 20% greater in these sublines; upon differentiation, though, they increase to levels similar to those of the mature parent cells. These data suggest that the dramatic changes seen in P-tyr, tyrosine kinase, and protein phosphotyrosine phosphatase activities seen with DMSO-induced maturation are specific for differentiation and are not an independent drug effect.

A cardinal feature of differentiation is the cessation of proliferation. The changes in P-tyr regulaton seen may reflect growth inhibition rather than differentiation per se. This possibility seemed unlikely, as cells which stop proliferating as they enter the plateau phase do not exhibit these changes. To further address this question, however, another class of compounds which induces granulocytic maturation was employed. The anthracyclines are a group of antibiotics with some activity against human tumors (106). Two members of this class of compounds, aclacinomycin A (ACM) and marcellomycin (MCM), induce granulocytic differentiation of HL-60 cells; a third, Adriamycin (ADR), lacks this ability (106). Concentrations of these three

TABLE II

Phosphoaminoacid Distribution of HL-60/DMSO-Y1 and Y2 Cells

Cell line	Treatment	P-Tyr	P-Ser	P-Thr
HL-60/DMSO-Y1	-RA	1.0 ± 0.2	89.4 ± 1.1	9.6 ± 0.4
	+RA	0.1 ± 0.1	89.7 ± 1.0	10.2 ± 0.3
HL-60/DMSO-Y2	-RA	1.1 ± 0.2	89.9 ± 1.0	9.0 ± 0.4
	+RA	0.1 ± 0.1	89.6 ± 0.9	10.3 ± 0.5

Percentages of phosphorylated amino acids represented by phosphotyrosine (P-Tyr), phosphoserine (P-Ser), and phosphothreonine (P-Thr). HL-60/DMSO-Y1 and Y2 cells were either untreated or treated with 10^{-6}M RA for 6 days, and the phosphoaminoacid distribution determined as described in the text. All cultures contained 1.2% (v/v) DMSO, which was necessary for cellular propagation. Values represent the mean ± standard deviation of 3 separate experiments.

Figure 37. Tyrosine kinase activity of HL-60/DMSO Y1 and Y2 cells treated with DMSO and RA. HL-60/DMSO Y1 (circles) and Y2 (triangles) were either untreated (open) or treated (filled) with 10^{-6} M RA, and tyrosine kinase of the particulate fractions determined. All cultures contained 1.2% (v/v) DMSO, which was necessary for cell propagation. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

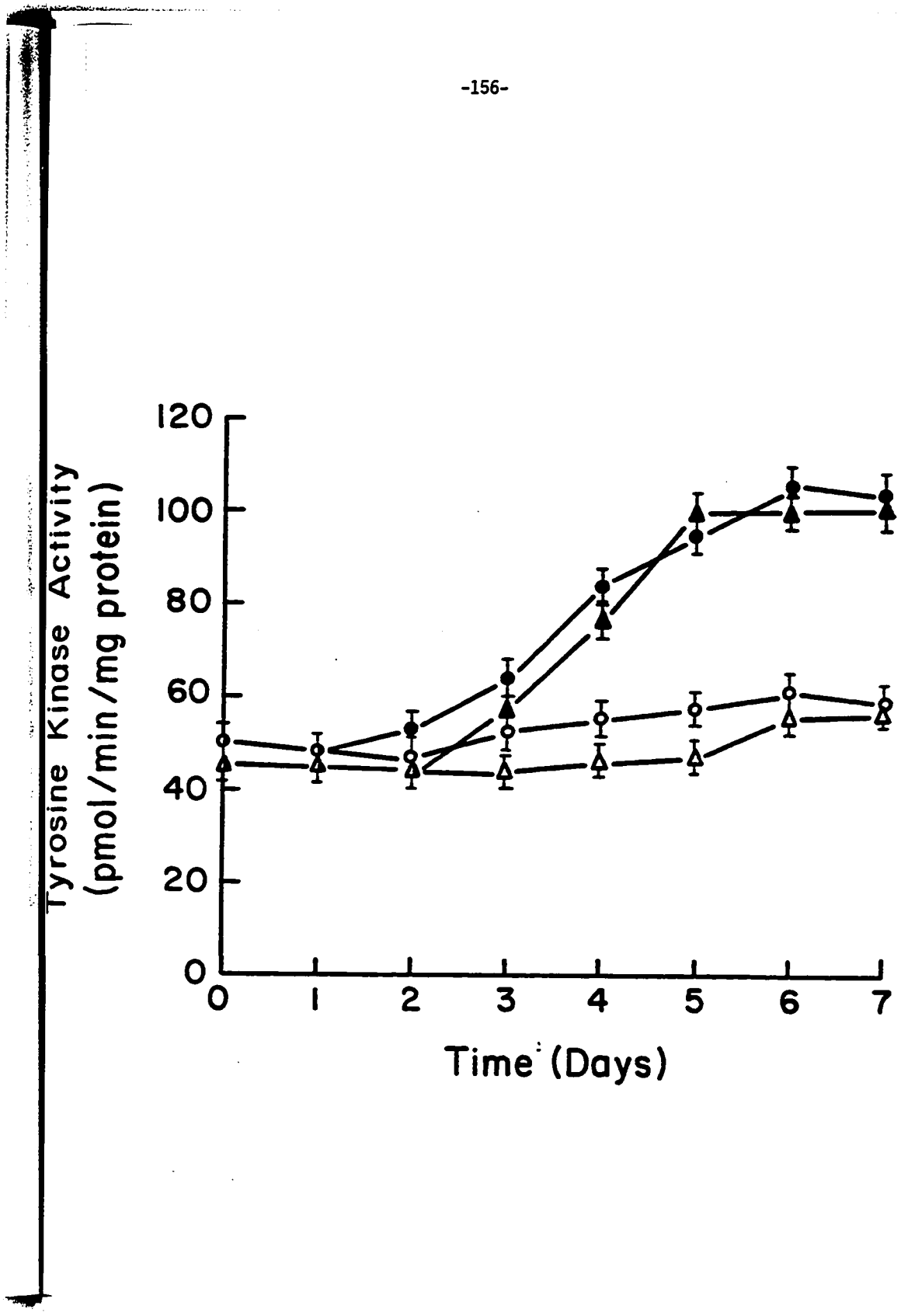


Figure 38A. Phosphotyrosine phosphatase activity of HL-60/DMSO Y1 and Y2 cells treated with DMSO and RA. HL-60/DMSO Y1 (circles) and Y2 (triangles) were either untreated (open) or treated (filled) with 10^{-6} M RA, and phosphotyrosine phosphatase activity determined. All cultures contained 1.2% (v/v) DMSO, which was necessary for cell propagation. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

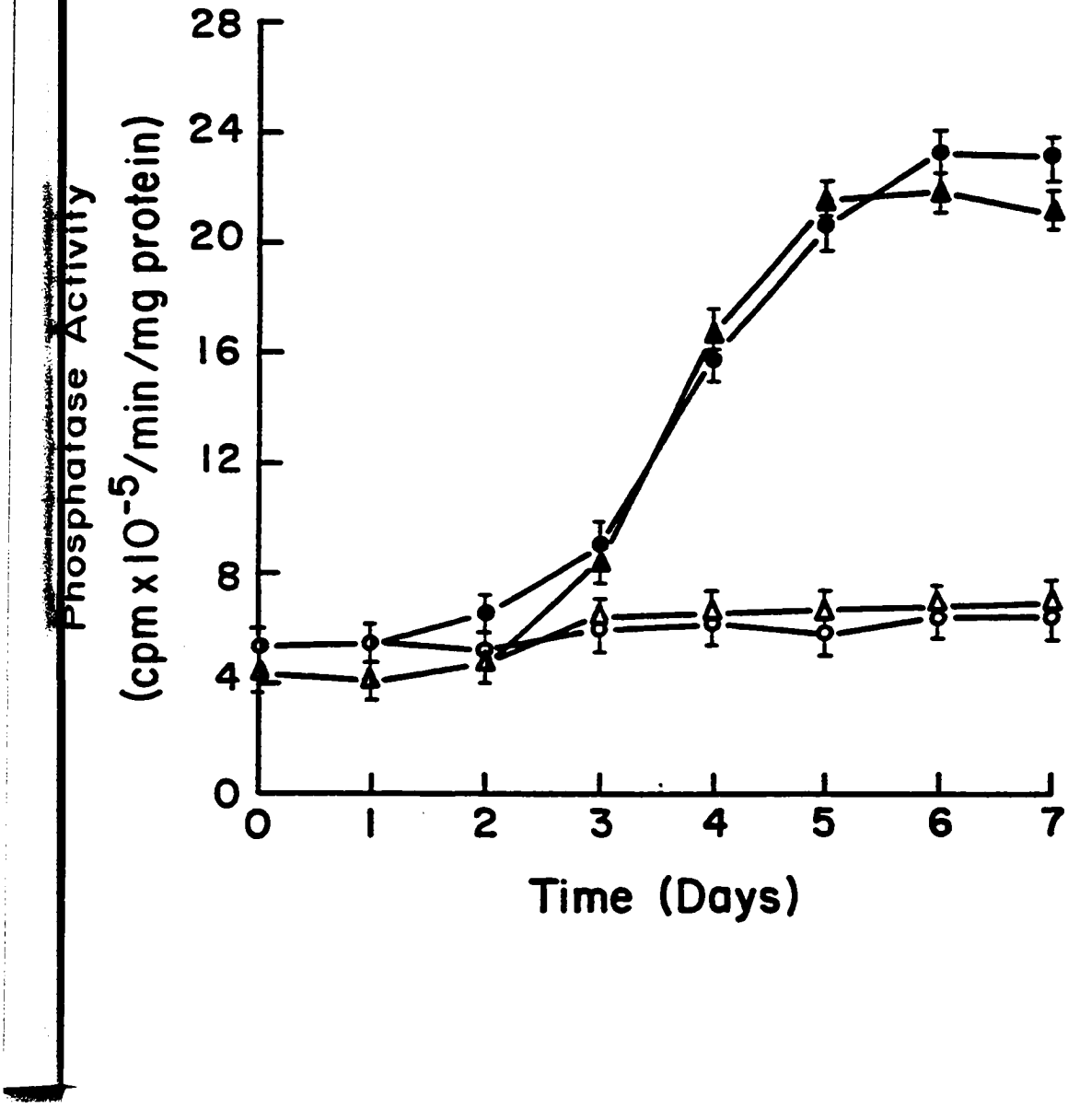
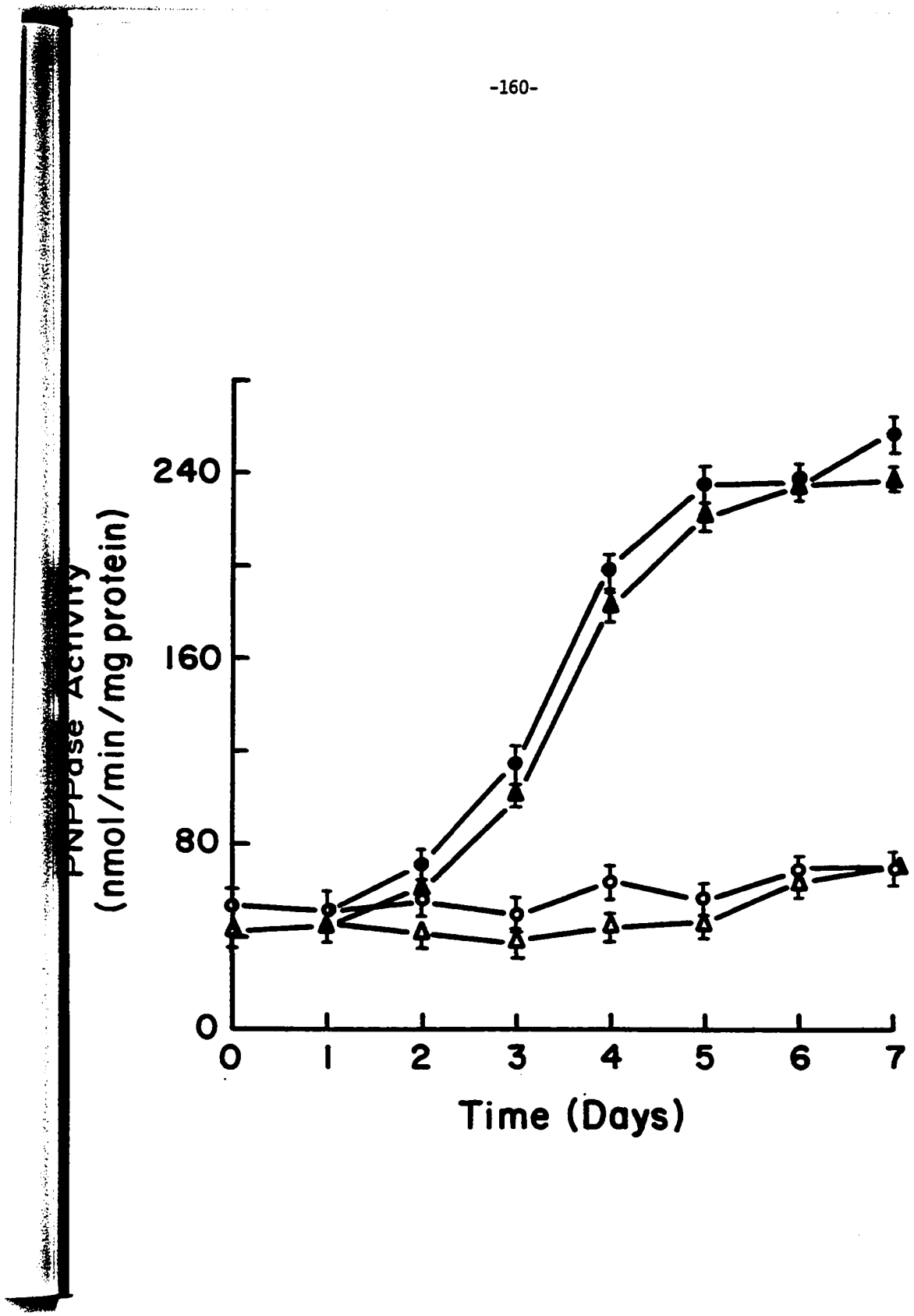


Figure 38B. PNPPase activity of HL-60/DMSO Y1 and Y2 cells treated with DMSO and RA. HL-60/DMSO Y1 (circles) and Y2 (triangles) were either untreated (open) or treated (filled) with 10^{-6} M RA, and PNPPase activity determined. All cultures contained 1.2% (v/v) DMSO, which was necessary for cell propagation. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.



drugs were employed which caused almost equal inhibition of growth (Figure 39), yet only ACM and MCM induced differentiation (Figure 40). Employing this system, it was found that only ACM and MCM caused a reduction in P-tyr levels (Table 12). Similarly, a two-fold increase in tyrosine kinase activity was seen with the agents that induced maturation, but not with ADR (Figure 41). Finally, ACM and MCM induced about a 4-fold increase in protein phosphotyrosine phosphatase (Figure 42) and PNPPase (Figure 43) activities, while the ADR-treated cells displayed only small increases in these activities. Thus, using these three related compounds, all of which inhibited growth to a similar extent, only those inducing differentiation caused profound changes in P-tyr regulation. In other experiments using equimolar concentrations of the three agents (40 nM), growth inhibition of ADR-treated cells was nearly complete, and the changes in the P-tyr indices were not significantly different (data not shown).

To further distinguish between changes due to differentiation and those secondary to cytotoxicity, a mutant HL-60 subline previously developed in this laboratory by Ishiguro et al. (144) was employed. These cells (designated HL-60/HGPRT$^-$) lack hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity and were derived by mutagenesis and selection (144). Whereas the parent cell line shows growth inhibition when treated with 10^{-6} M of the

Figure 39. Growth of HL-60 cells treated with anthracyclines. HL-60 cells were treated with 25 nM ADR (Δ), 40 nM MCM (o), 50 nM ACM (▲), or untreated (●), and cell number was determined as described. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

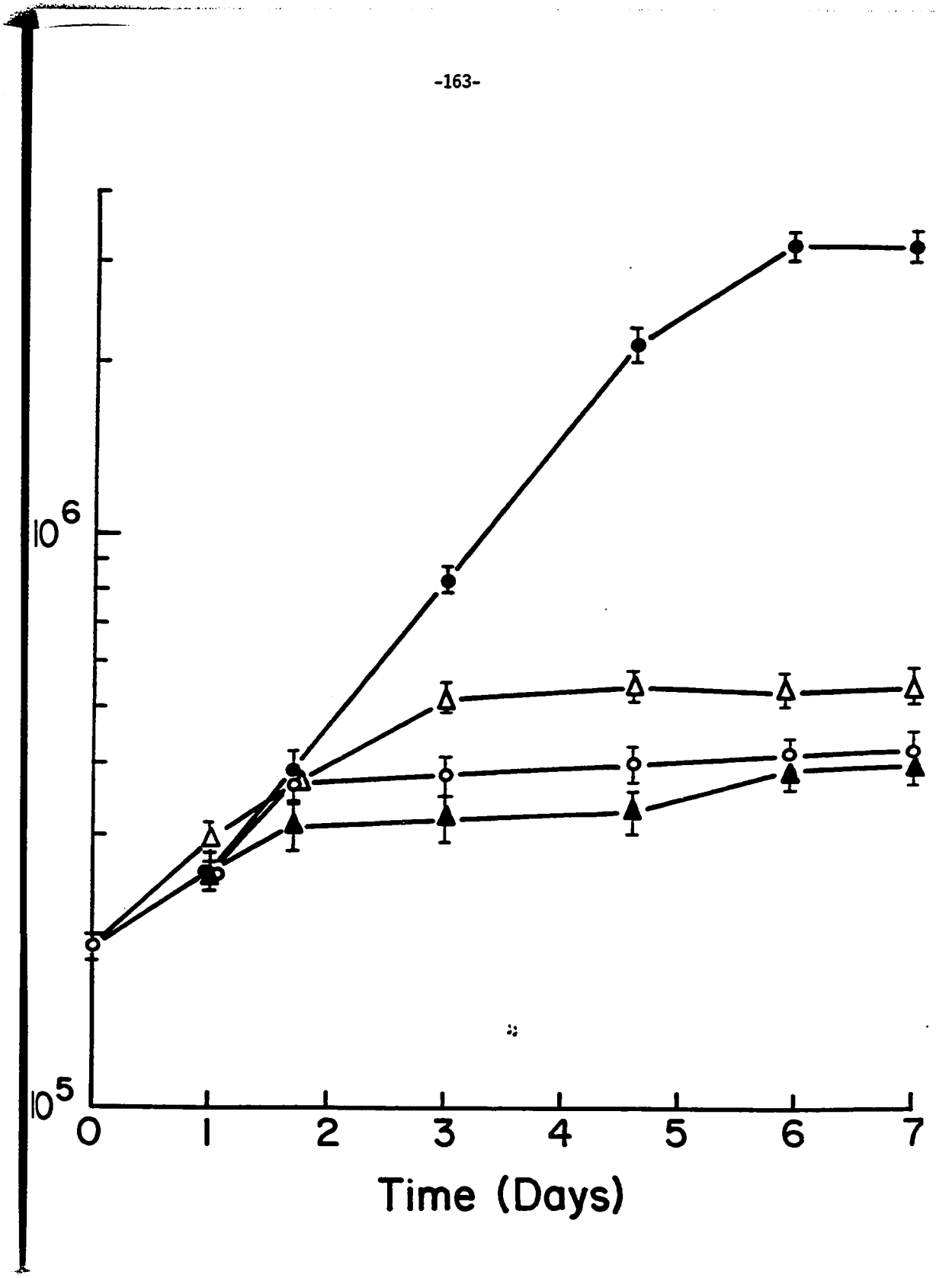


Figure 40. Differentiation of HL-60 cells treated with anthracyclines. HL-60 cells were treated with 25 nM ADR (Δ), 40 nM MCM (○), 50 nM ACM (▲), or untreated (●), and the ability to reduce nitroblue tetrazolium was assayed. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

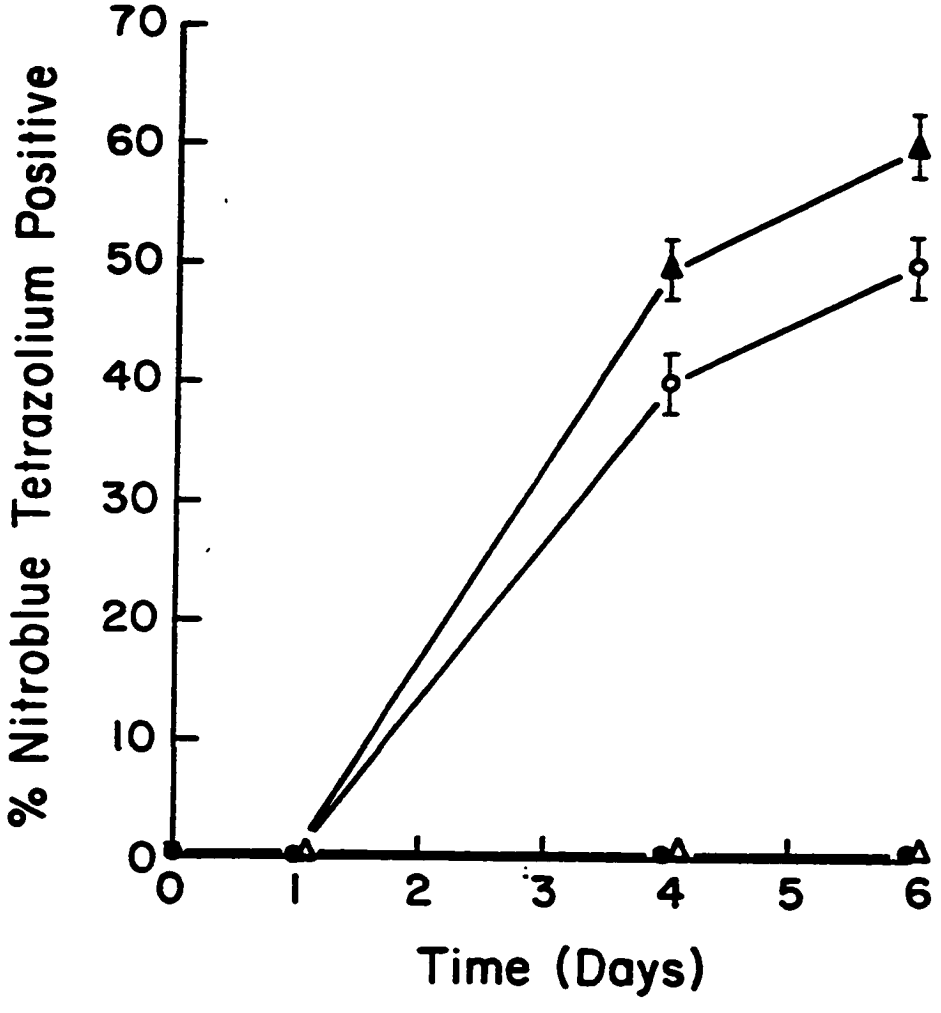


TABLE 12

Phosphoaminoacid Distribution of HL-60 Cells Treated with Anthracyclines

Treatment	P-Tyr	P-Ser	P-Thr
None	1.5 ± 0.2	89.7 ± 0.9	8.8 ± 0.3
ACM (50 nM)	0.3 ± 0.1	90.3 ± 1.1	9.4 ± 0.4
MCM (40 nM)	0.2 ± 0.1	90.1 ± 1.0	9.7 ± 0.4
ADR (25 nM)	1.3 ± 0.2	89.9 ± 1.0	8.8 ± 0.3

Percentages of phosphorylated amino acids represented by phosphotyrosine (P-Tyr), phosphoserine (P-Ser), and phosphothreonine (P-Thr). HL-60 cells were treated with the indicated drug for 6 days, and the phosphoaminoacid distribution determined as described in the text. Values represent the mean ± standard deviation of 3 separate experiments.

Figure 41. Tyrosine kinase activity of HL-60 cells treated with anthracyclines. HL-60 cells were treated with 25 nM ADR (Δ), 40 nM MCM (○), 50 nM ACM (▲), or untreated (●), and tyrosine kinase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

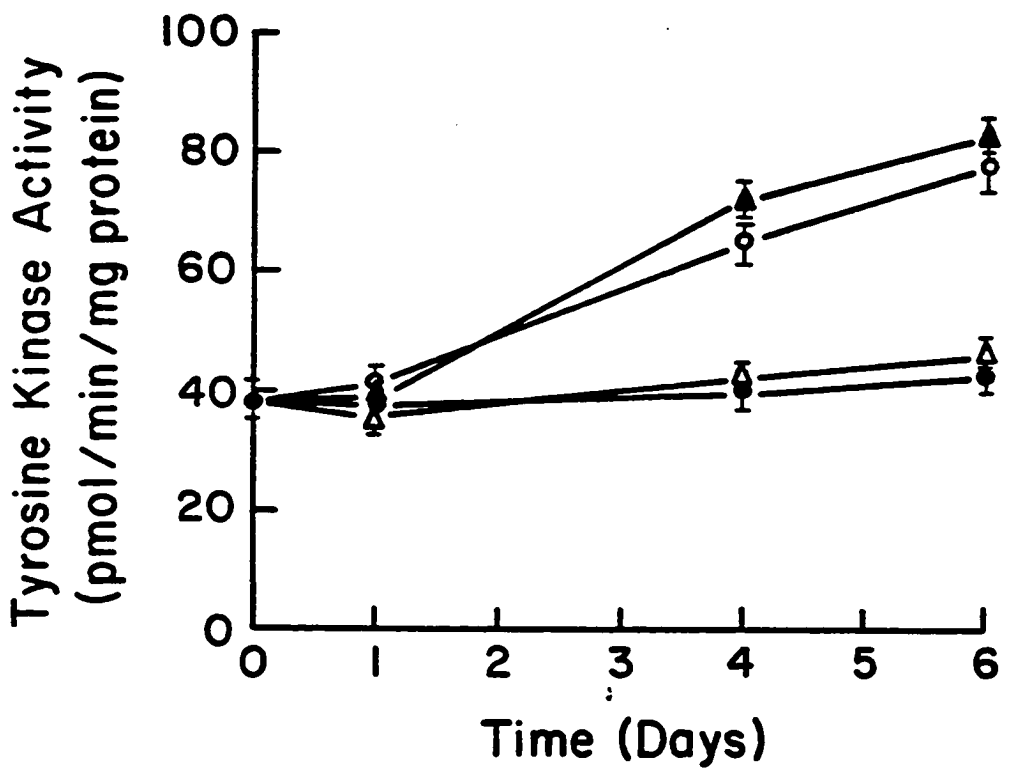


Figure 42. Phosphotyrosine phosphatase activity of HL-60 cells treated with anthracyclines. HL-60 cells were treated with 25 nM ADR (Δ), 40 nM MCM (○), 50 nM ACM (▲), or untreated (●), and phosphotyrosine phosphatase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

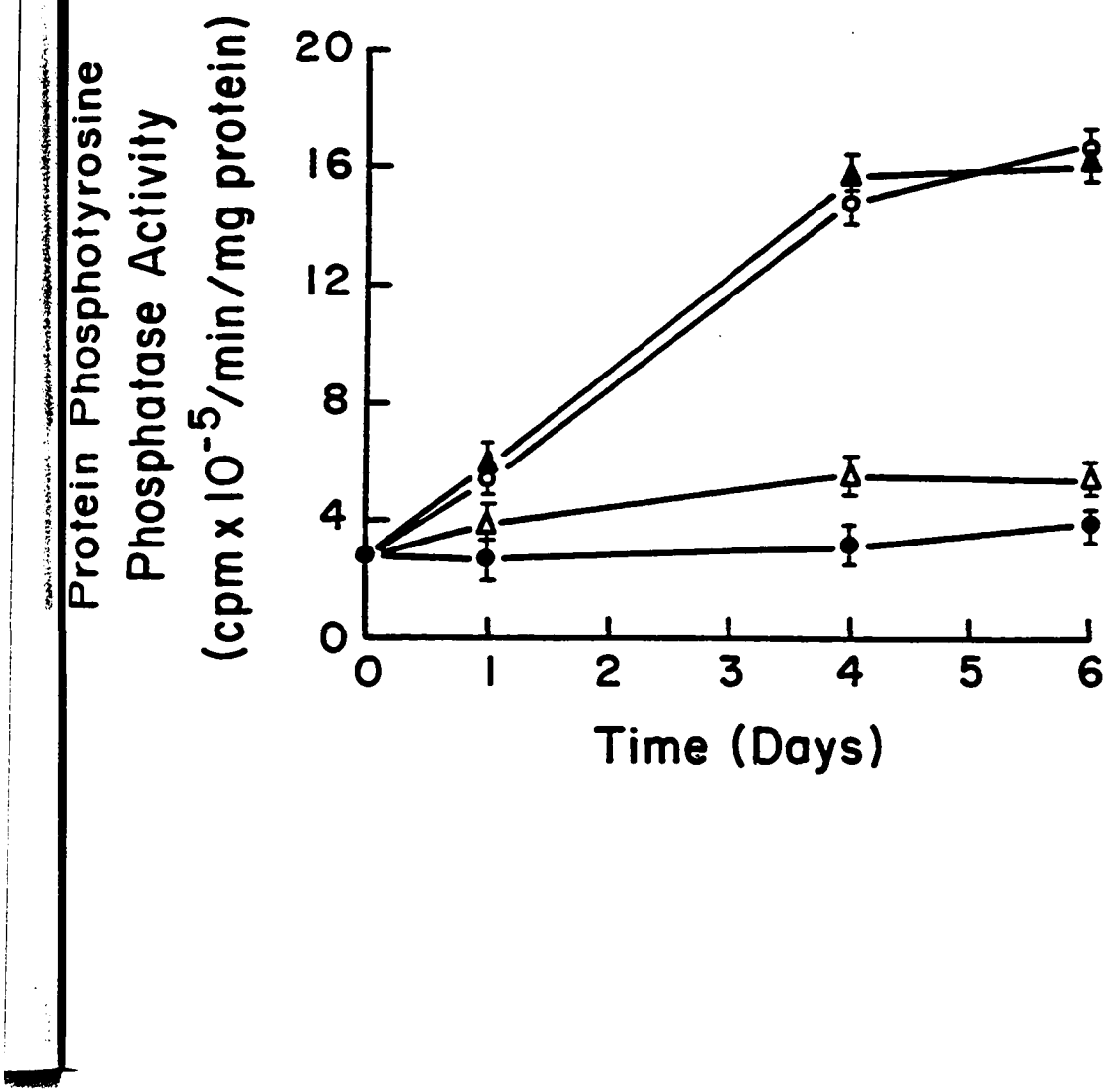
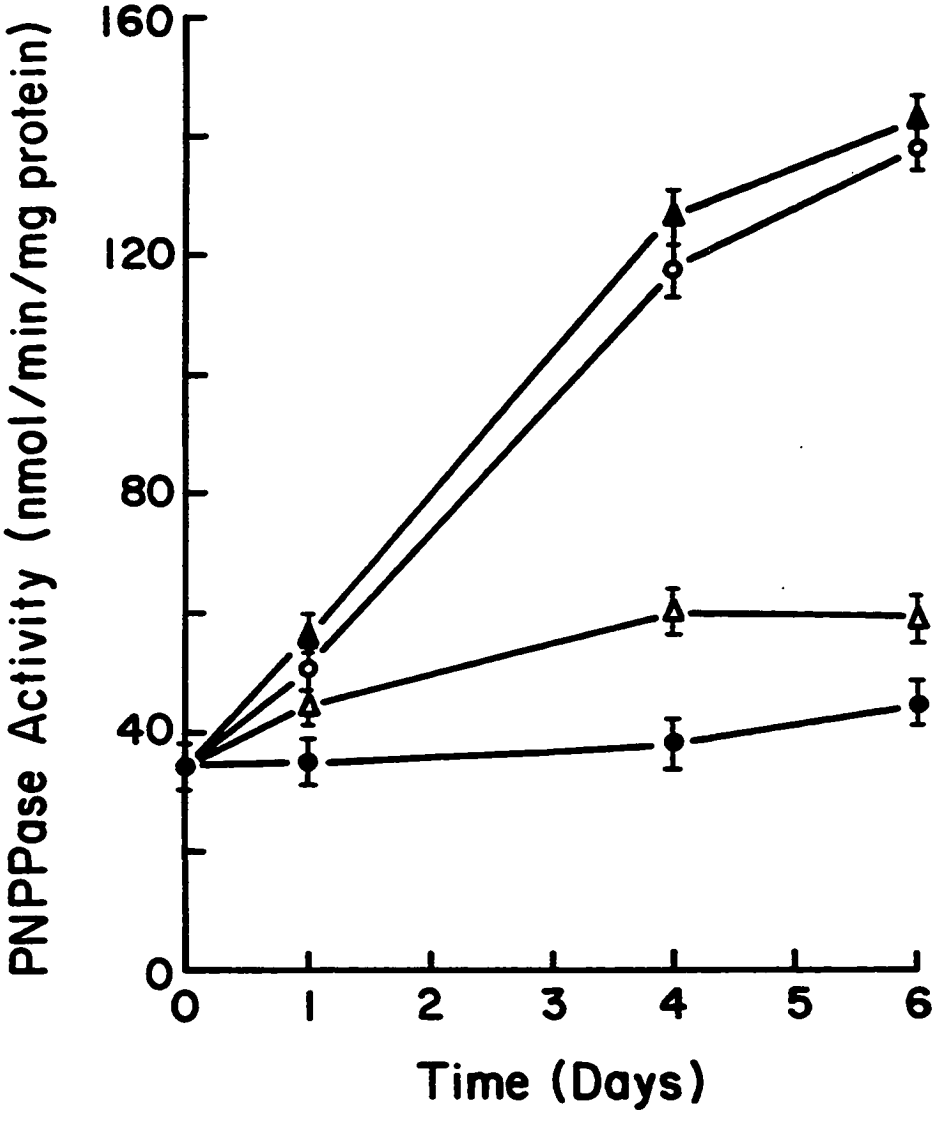


Figure 43. PNPPase activity of HL-60 cells treated with anthracyclines. HL-60 cells were treated with 25 nM ADR (Δ), 40 nM MCM (O), 50 nM ACM (▲), or untreated (●), and PNPPase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.



purine analog 6-thioguanine (TG), these mutant cells are inhibited to the same extent only at the much higher concentration of 3×10^{-4} M (Figure 44); untreated HL-60/HGPRT$^-$ grow at a similar rate as the parent line. When treated at these concentrations, HL-60 cells show no differentiation; 65% of the mutant line, however, are functionally mature after seven days (Figure 45). Thus, using these two cell lines and a single agent, the effects of differentiation and cytotoxicity can again be dissected. As shown in Table 13, only the differentiating HL-60/HGPRT$^-$ cells display a fall in P-tyr content. In examining tyrosine kinase activity it was found that the mutant line possessed approximately 30% greater activity; upon treatment with TG, this activity increased 2.3-fold; no change was seen when the parent line was exposed to TG (Figure 46). In an analogous manner, protein phosphotyrosine phosphatase (Figure 47A) and PNPPase (Figure 47B) activities were similar in the untreated and mutant lines; upon treatment with TG, however, these activities increased by about 4-fold in the mutant, and remained unchanged in the parent line.

Changes in phosphotyrosine regulation during the granulocytic differentiation of WEHI-3B leukemia cells. The general pattern established with the granulocytic maturation of HL-60 cells is that a decrease in P-tyr of an order of magnitude occurs, accompanied by an increase of 2- to 4-fold in tyrosine kinase activity and 6- to 8-fold in

Figure 44. Growth of HL-60 and HL-60/HGPRT$^-$ cells treated with TG. HL-60 (circles) and HL-60/HGPRT$^-$ (triangles) cells were either untreated (open) or treated (filled) with 1 uM and 300 uM TG, respectively, and cell number determined. Viability was consistently greater than 90%. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

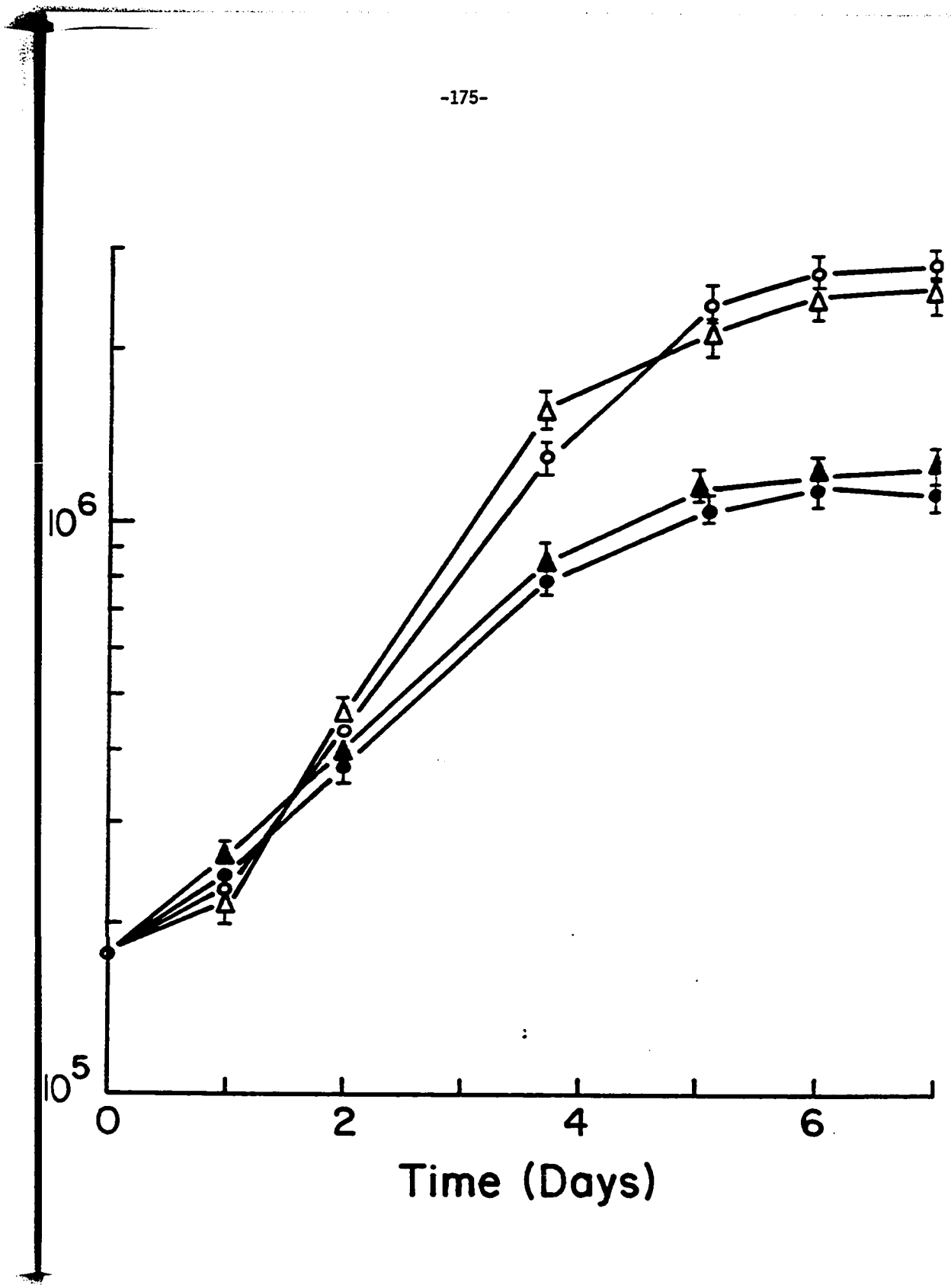


Figure 45. Differentiation of HL-60 and HL-60/HGPRT$^-$ cells treated with TG. HL-60 (circles) and HL-60/HGPRT$^-$ (triangles) cells were either untreated (open) or treated (filled) with 1 uM and 300 uM TG, respectively, and the ability to reduce nitroblue tetrazolium was assessed. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

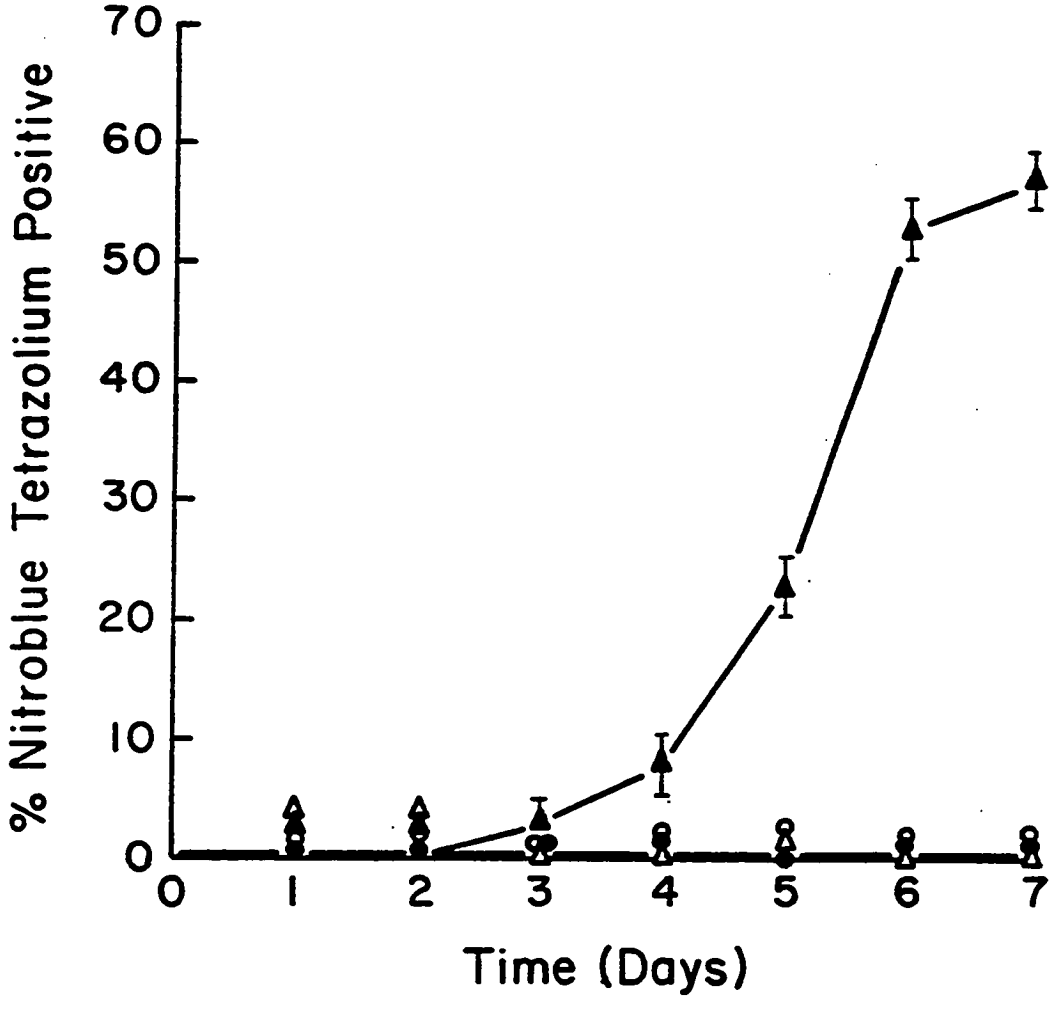


TABLE 13

Phosphoaminoacid Distribution of HL-60 and HL-60/HGPRT$^-$ Cells Treated with TG

Cell line	TG	P-Tyr	P-Ser	P-Thr
HL-60	—	1.5 ± 0.2	88.7 ± 0.9	9.8 ± 0.4
HL-60	1 μM	1.3 ± 0.2	89.2 ± 1.0	9.5 ± 0.4
HL-60/HGPRT$^-$	—	1.4 ± 0.2	88.9 ± 1.0	9.7 ± 0.5
HL-60/HGPRT$^-$	300 μM	0.2 ± 0.1	89.4 ± 0.8	10.4 ± 0.6

Percentages of phosphorylated amino acids represented by phosphotyrosine (P-Tyr), phosphoserine (P-Ser), and phosphothreonine (P-Thr). HL-60 or HL-60/HGPRT$^-$ cells were either untreated or treated with TG, and the phosphoaminoacid distribution determined as described in the text. Values represent the mean ± standard deviation of 3 separate experiments.

Figure 46. Tyrosine kinase activity of HL-60 and HL-60/HGPRT$^-$ cells treated with TG. HL-60 (circles) and HL-60/HGPRT$^-$ (triangles) cells were either untreated (open) or treated (filled) with 1 uM and 300 uM TG, respectively, and tyrosine kinase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

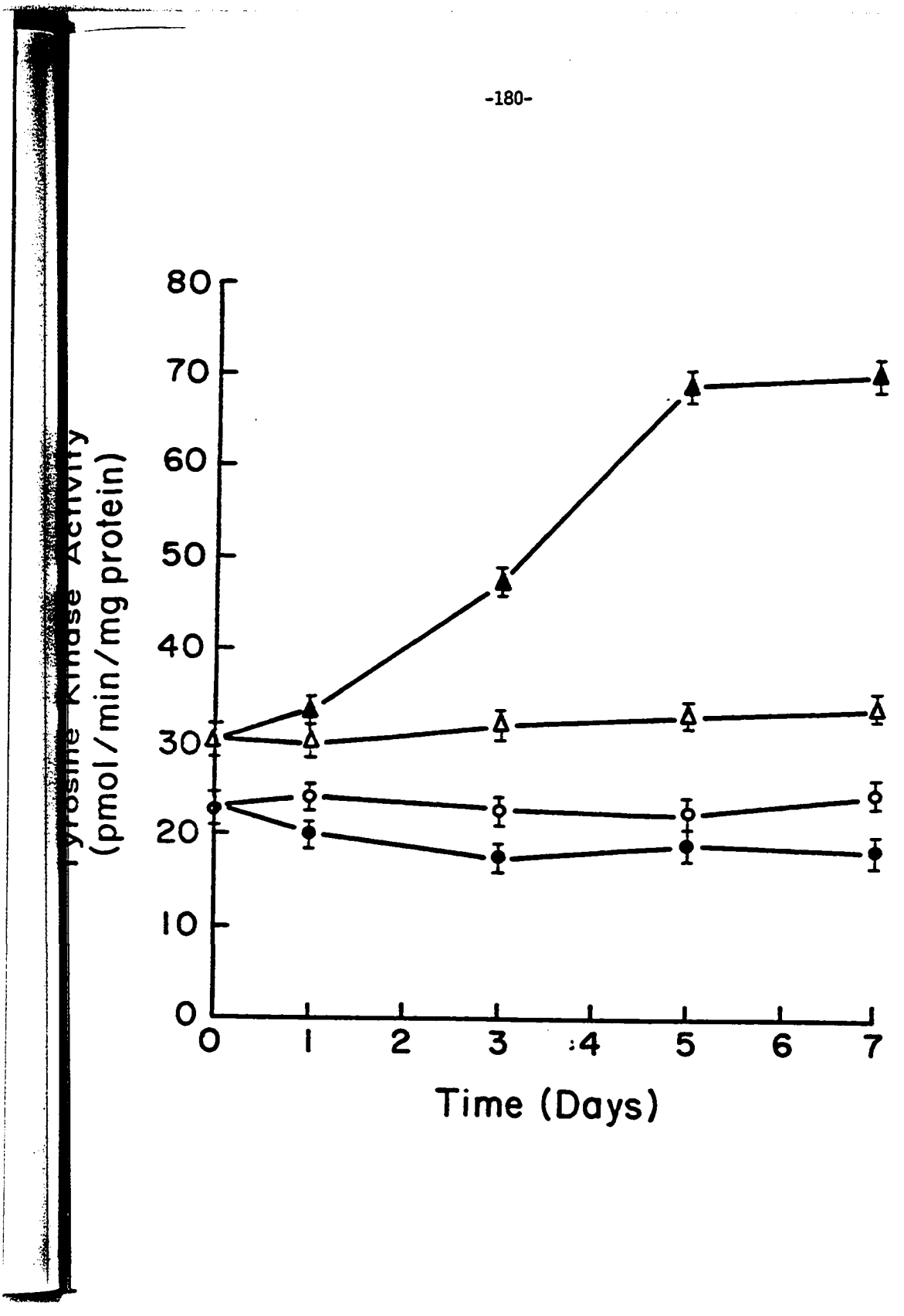


Figure 47A. Phosphotyrosine phosphatase activity of HL-60 and HL-60/HGPRT$^-$ cells treated with TG. HL-60 (circles) and HL-60/HGPRT$^-$ (triangles) cells were either untreated (open) or treated (filled) with 1 uM and 300 uM TG, respectively, and phosphotyrosine phosphatase activity was assessed. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

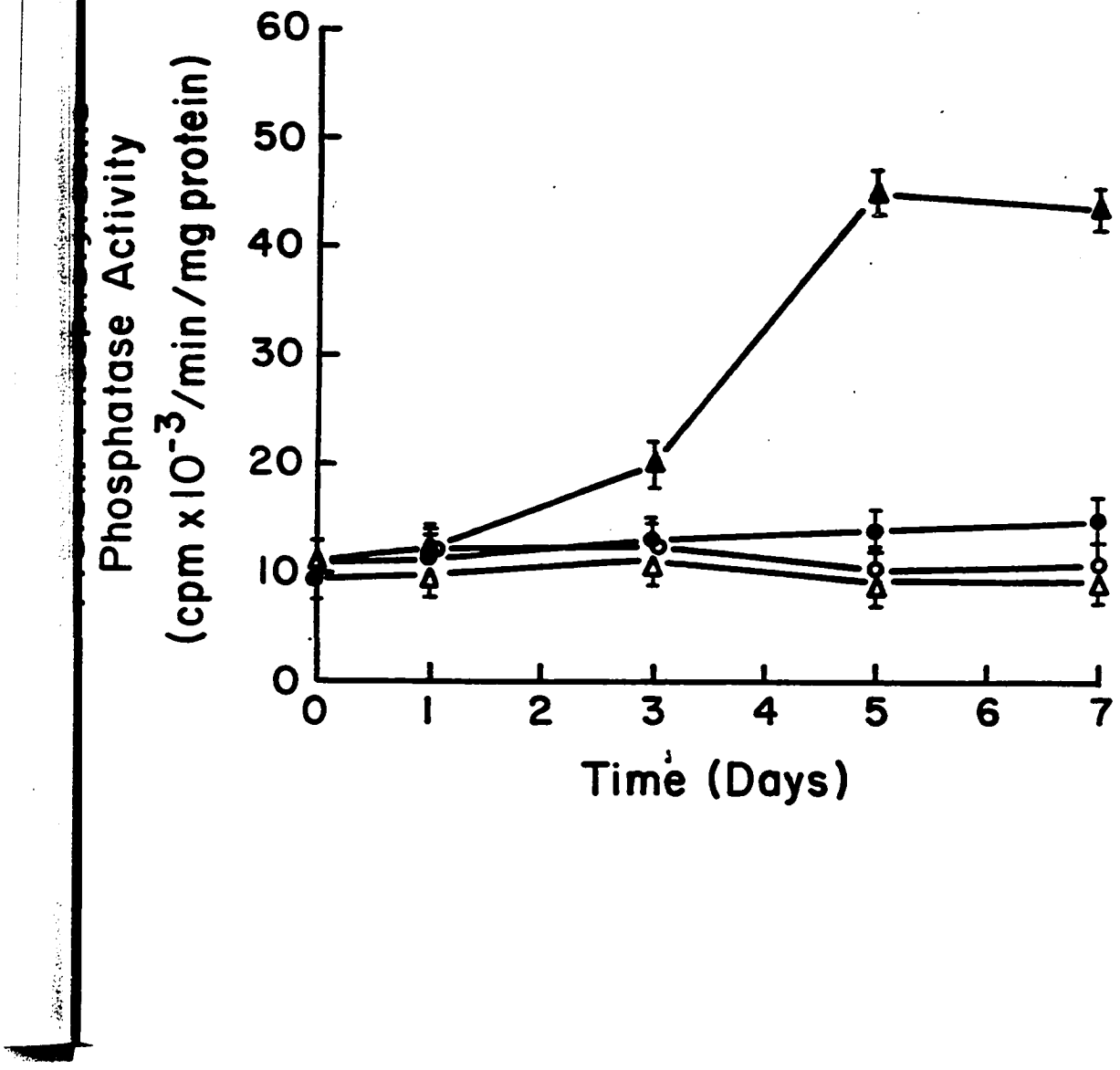
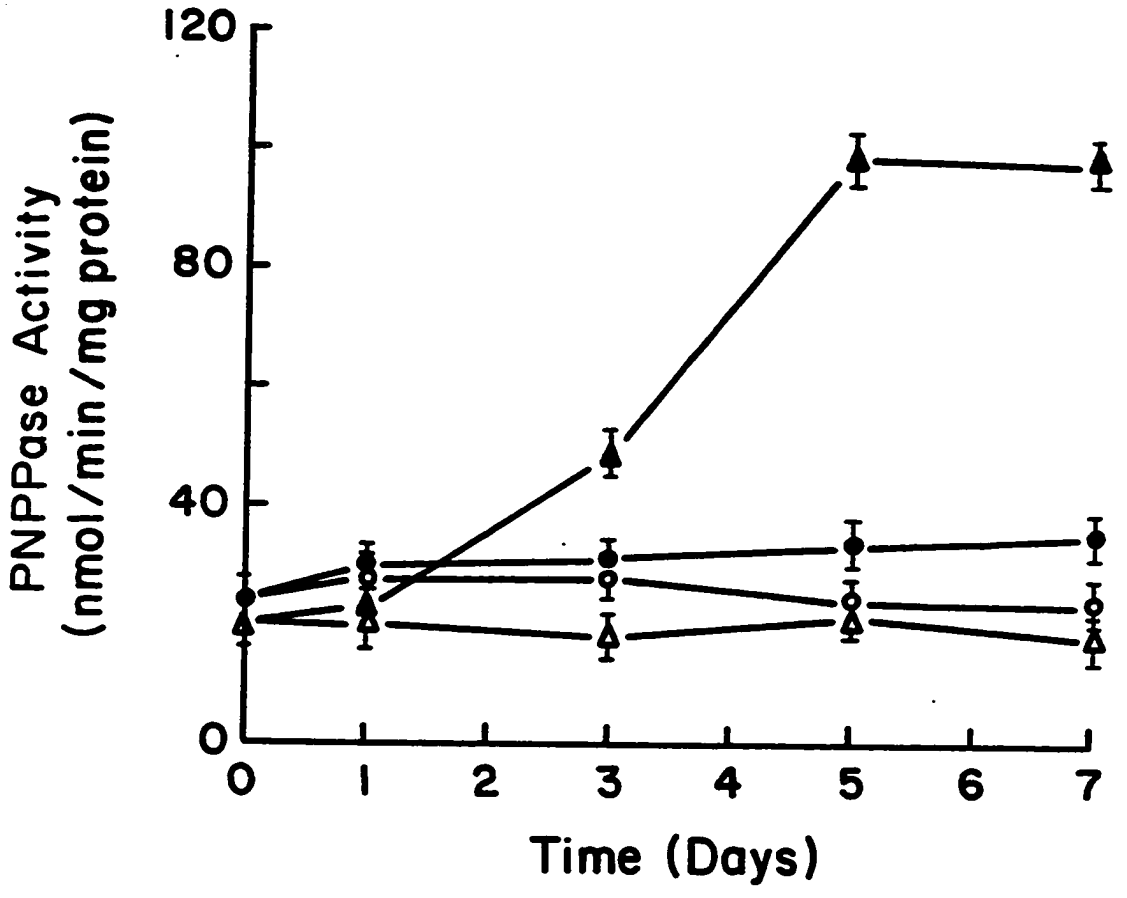


Figure 47B. PNPPase activity of HL-60 and HL-60/HGPRT$^-$ cells treated with TG. HL-60 (circles) and HL-60/HGPRT$^-$ (triangles) cells were either untreated (open) or treated (filled) with 1 uM and 300 uM TG, respectively, and PNPPase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.



phosphotyrosine phosphatase activity. In order to assess whether similar changes occur in a different system, WEHI-3B murine myelomonocytic leukemia cells were employed. This leukemia was developed in the BALB/c mouse (145) and can be induced to differentiate into mature granulocytes *in vitro* by the anthracyclines ACM, MCM, and, in contrast to HL-60, ADR (146). These cells grow with a doubling time of 9 hours; treatment with the anthracyclines leads to a profound inhibition of cell growth (Figure 48). Consistent with the more rapid growth rate of these cells, the anthracyclines induce maximal differentiation of these cells in 3 days (compared to 6 days for HL-60) (Figure 49); 55 to 67% of the treated cells are able to reduce NBT. Untreated WEHI-3B cells contain 1.2% P-tyr among their phosphoaminoacids; granulocytic differentiation causes a reduction to 0.1 to 0.2% (Table 14). Tyrosine kinase increases by 3- to 4-fold with differentiations, while cells which enter the plateau phase show no significant change (Figure 50). Basal tyrosine kinase activity is about one-fourth that in untreated HL-60 cells. As with the human cells, phosphotyrosine phosphatase (Figure 51) and PNPPase (Figure 52) activities rose as well with maturation, from 3- to 5-fold. Such increases were not observed in plateau phase cells. Untreated WEHI-3B cells possess approximately 70% the phosphotyrosine phosphatase activity of HL-60 cells. Thus it appears that in myelomonocytic leukemias from two species, similar

Figure 48. Growth of WEHI-3B cells treated with anthracyclines. WEHI-3B cells were treated with 40 nM ADR (○), 30 nM MCM (△), 30 nM ACM (▲), or untreated (●), and cell number was determined. Viability was consistently greater than 90%. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

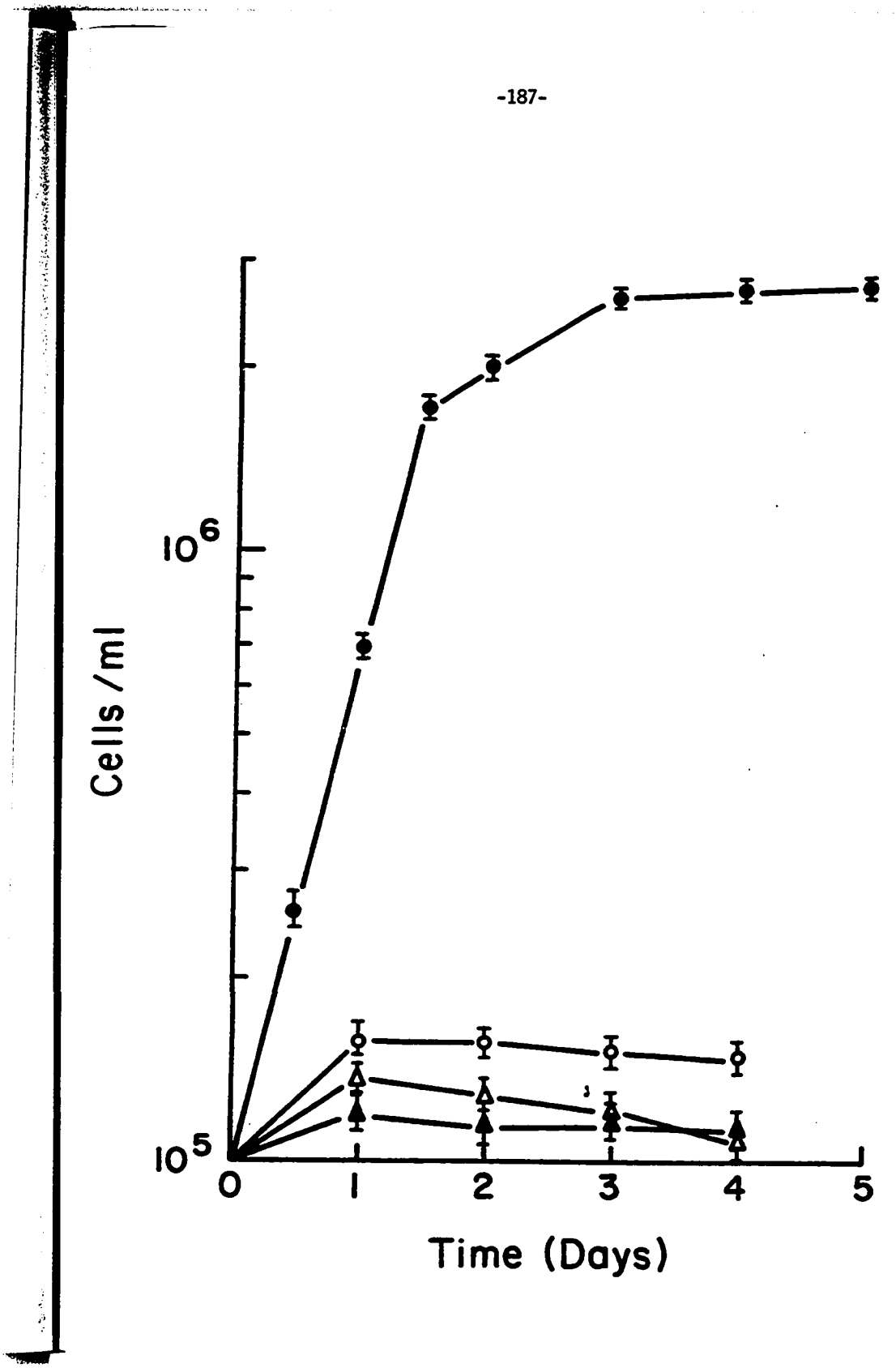


Figure 49. Differentiation of WEHI-3B cells treated with anthracyclines. WEHI-3B cells were treated with 30 nM ADR (○), 30 nM MCM (△), 30 nM ACM (▲), or untreated (●), and the ability to reduce nitroblue tetrazolium was assessed. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

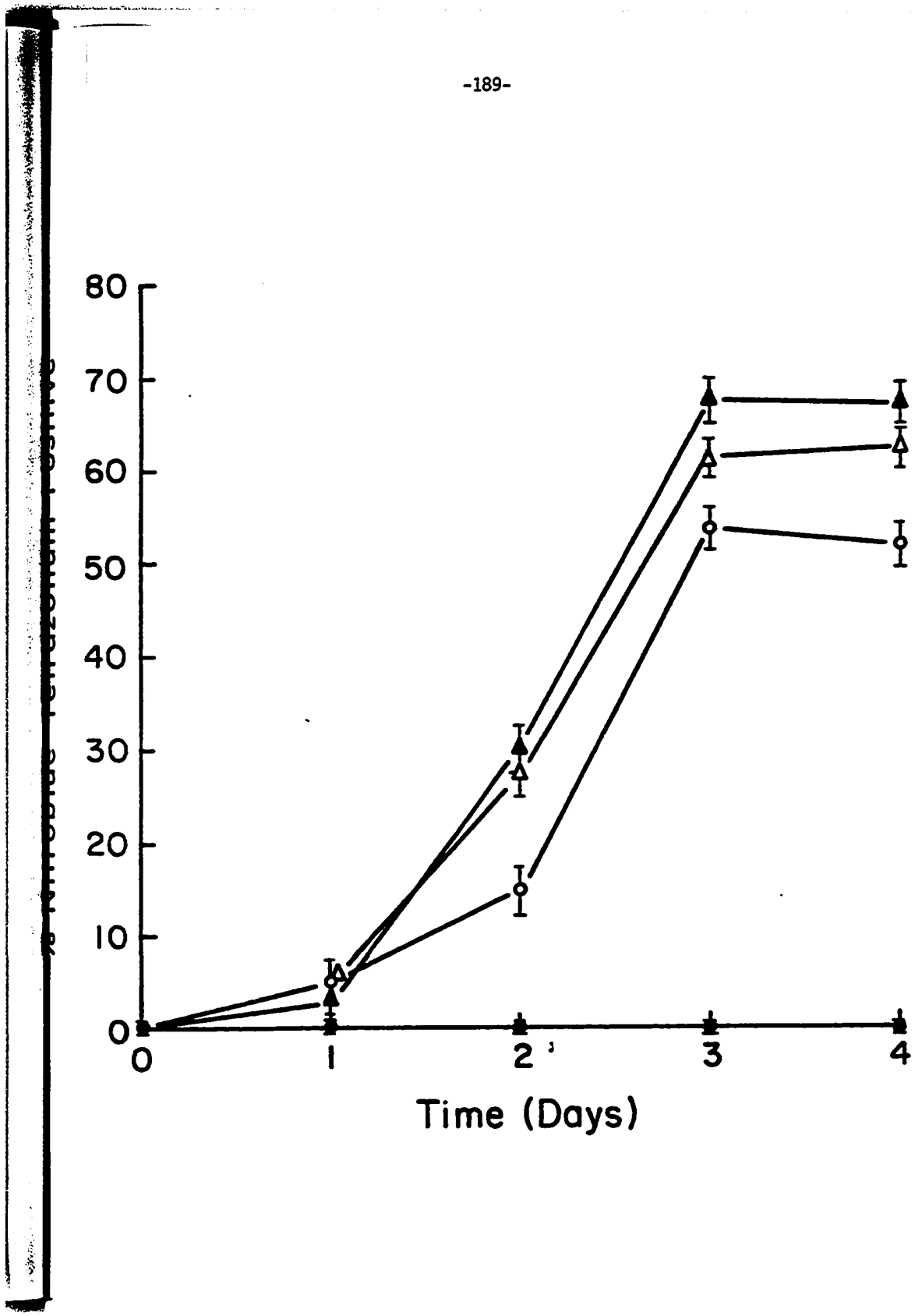


TABLE 14

Phosphoaminoacid Distribution of WEHI-3B Cells Treated with Anthracyclines

Treatment	P-Tyr	P-Ser	P-Thr
None (log phase)	1.2 ± 0.2	91.3 ± 1.0	7.5 ± 0.4
None (plateau phase)	1.1 ± 0.2	91.5 ± 1.0	7.4 ± 0.4
ACM (30 nM)	0.1 ± 0.1	92.0 ± 1.1	7.7 ± 0.5
ADR (40 nM)	0.1 ± 0.1	92.1 ± 1.2	7.8 ± 0.5
MCM (30 nM)	0.2 ± 0.1	91.8 ± 1.2	8.0 ± 0.5

Percentages of phosphorylated amino acids represented by phosphotyrosine (P-Tyr), phosphoserine (P-Ser), and phosphothreonine (P-Thr). WEHI-3B cells were treated with the indicated agent for 3 days, and the phosphoaminoacid distribution was determined as described in "Materials and Methods". Values represent the mean ± standard deviation of 3 separate determinations.

Figure 50. Tyrosine kinase activity of WEHI-3B cells treated with anthracyclines. WEHI-3B cells were treated with 40 nM ADR (○), 30 nM MCM (△), 30 nM ACM (▲), or untreated (●), and tyrosine kinase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

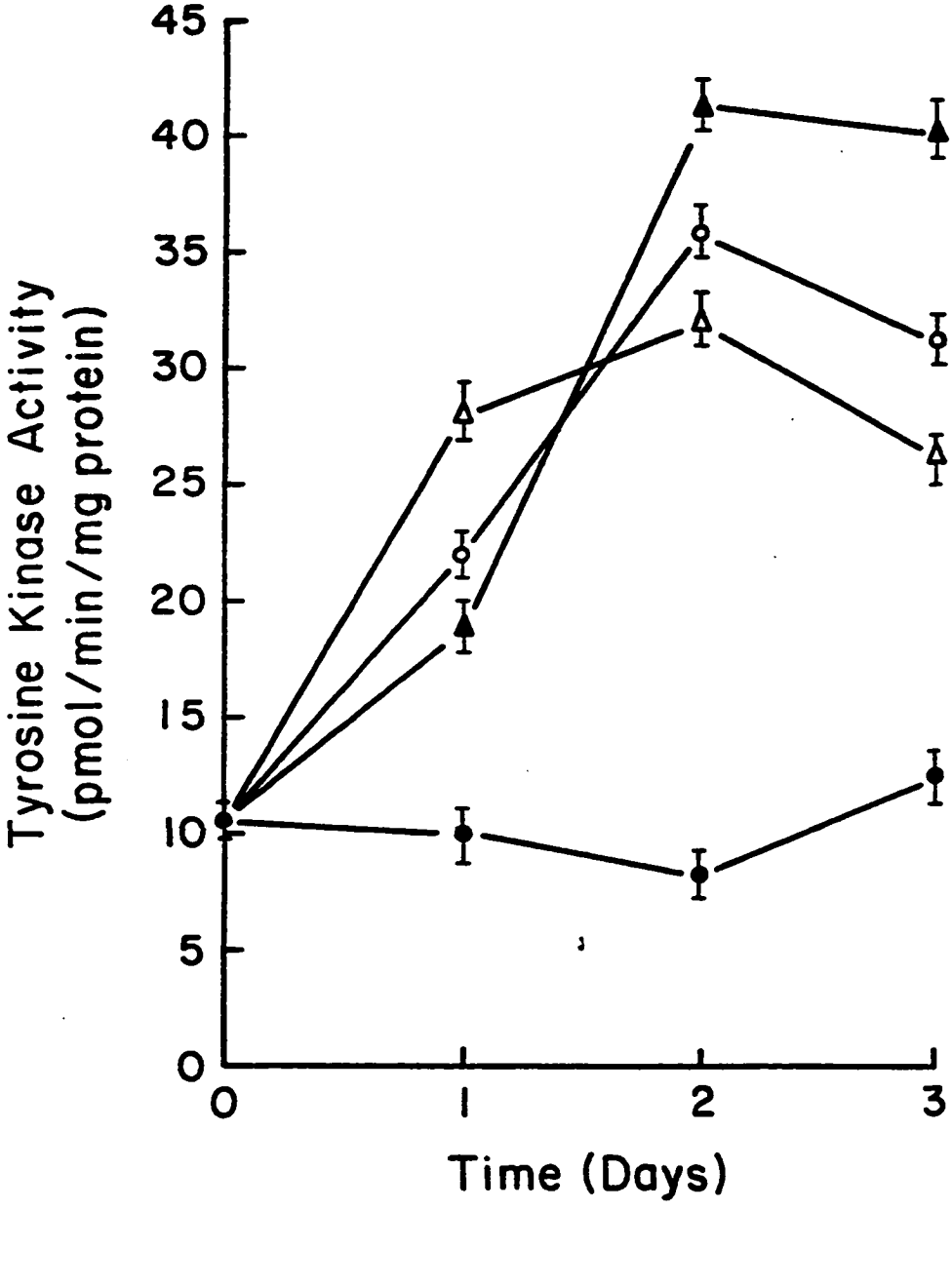


Figure 51. Phosphotyrosine phosphatase activity of WEHI-3B cells treated with anthracyclines. WEHI-3B cells were treated with 40 nM ADR (○), 30 nM MCM (△), 30 nM ACM (▲), or untreated (●), and phosphotyrosine phosphatase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

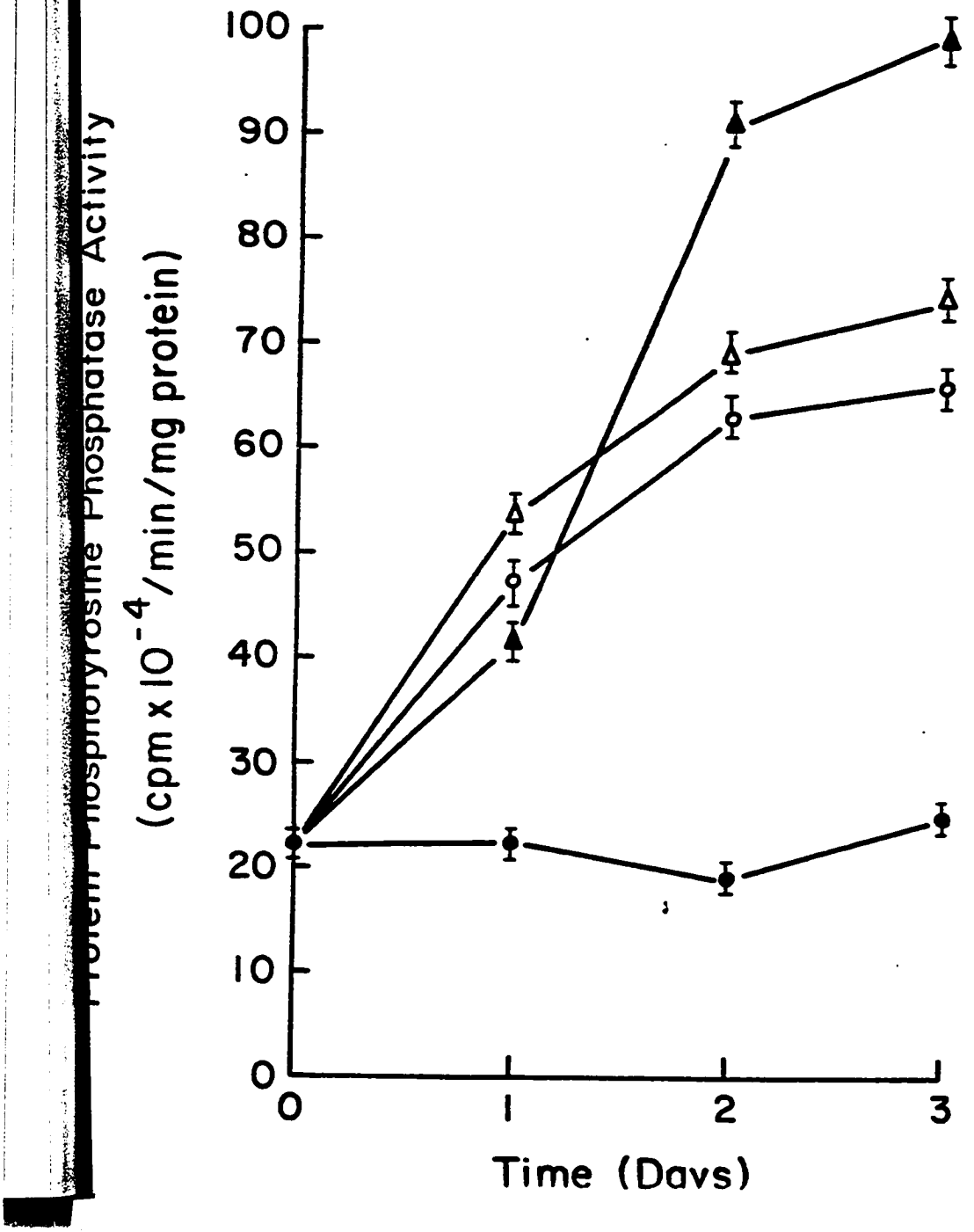
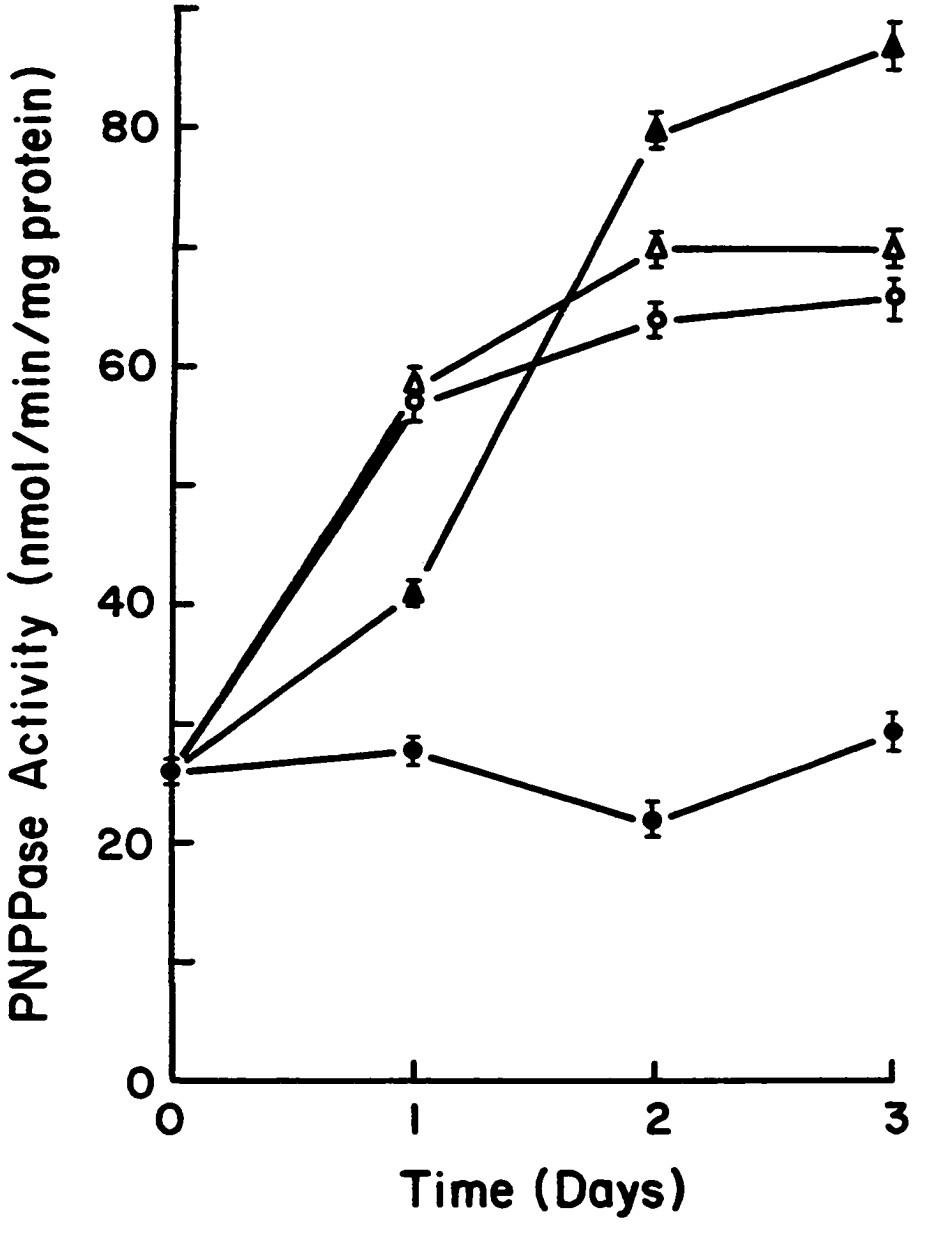


Figure 52. PNPPase activity of WEHI-3B cells treated with anthracyclines. WEHI-3B cells were treated with 40 nM ADR (○), 30 nM MCM (△), 30 nM ACM (▲), or untreated (●), and PNPPase activity was assayed. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.



qualitative changes in phosphotyrosine metabolism occur. It is also significant that ADR induces these changes in the WEHI-3B cells which differentiate with ADR and not in HL-60 cells which do not differentiate in respone to ADR. This lends support to the theory that these changes are specific for the differentiation process, and are not secondary to other types of drug effects.

Changes in phosphotyrosine regulation in HL-60 cells during monocytic differentiation. Given the dramatic changes in the enzymes which regulate tyrosine phosphorylation that attend granulocytic differentiation, experiments were conducted to examine the changes that accompany monocytic maturation. HL-60 cells provide a unique opportunity to study true differentiation, given their bipotent nature, rather than the mere progression of a cell along a precommitted pathway. The phorbol ester TPA induces monocytic differentiation of HL-60 cells (110). At a concentration of 10^{-8} M, 50 to 60% of the population exhibits the monocytic marker of non-specific esterase activity (Figure 53). TPA is also known to stimulate protein kinase C in these cells (111), however, this does not appear to be the sole inducing step. The diacylglycerol analog OAG also stimulates protein kinase C in HL-60 cells, yet it fails to induce differentiation (111). Monocytic differentiation of HL-60 cells leads to a large decline in P-tyr content (Table 15), while OAG treatment causes no

Figure 53. Differentiation of HL-60 cells treated with TPA and OAG. HL-60 cells were treated with 10^{-8} M TPA (○), 40 ug/ml OAG (△), or untreated (●), and the ability to hydrolyze alpha-naphthyl acetate was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

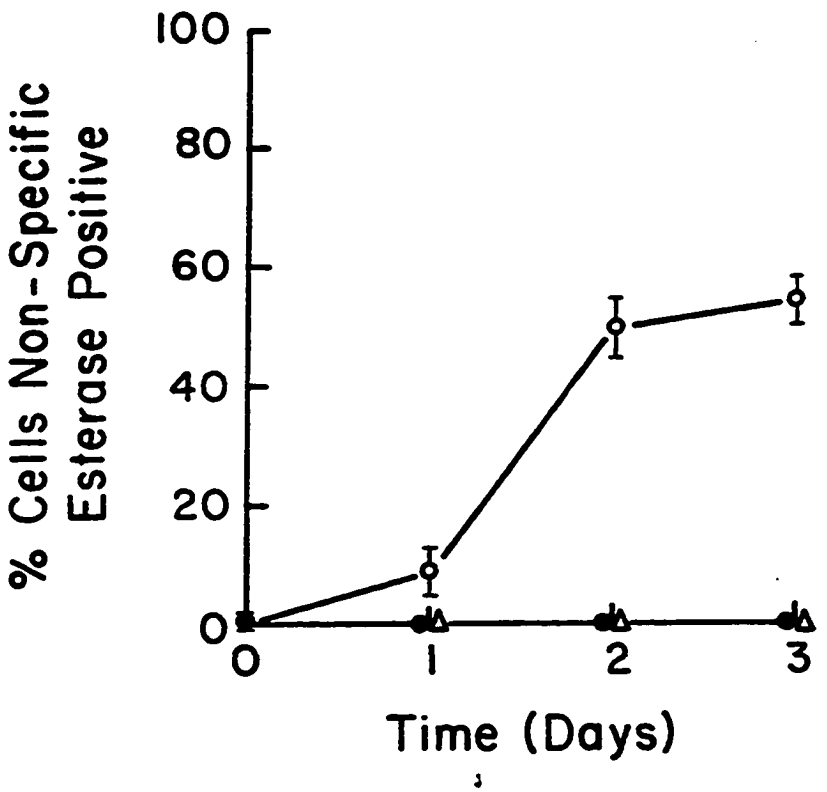


TABLE 15

Phosphoaminoacid Distribution of HL-60 Cells Treated with TPA and OAG

Treatment	P-Tyr	P-Ser	P-Thr
None	1.5 ± 0.2	88.4 ± 0.9	10.1 ± 0.4
TPA (10^{-8}M)	0.1 ± 0.1	89.1 ± 0.9	10.8 ± 0.5
OAG (40 µg/ml)	1.5 ± 0.2	88.6 ± 0.8	9.9 ± 0.4

Percentages of phosphorylated amino acids represented by phosphotyrosine (P-Tyr), phosphoserine (P-Ser), and phosphothreonine (P-Thr). HL-60 cells were treated with the indicated agent for 3 days, and the phosphoaminoacid distribution was determined as described in in the text. Values represent the mean ± standard deviation of 3 separate determinations.

change. Such maturation is also accompanied by a 2-fold increase in tyrosine kinase activity (Figure 54) and an 8- to 10-fold increase in protein phosphotyrosine phosphatase (Figure 55) and PNPPase (Figure 56) activities. No such changes are seen in OAG-treated cells. Parallel experiments performed in this laboratory (employing the same stocks of cells, OAG, and TPA) demonstrated that OAG increased protein kinase C activity to the same extent as TPA at the concentrations used (111). Thus, qualitatively similar changes in P-tyr content and tyrosine kinase and phosphotyrosine phosphatase activities accompany both granulocytic and monocytic differentiation of HL-60 cells.

Comparison of enzyme activities in differentiated and undifferentiated HL-60 cells. Although tyrosine kinase and phosphotyrosine phosphatase activities increase in differentiated HL-60 cells, it is unclear whether this reflects increased synthesis of the same or new enzymes, decreased degradation of enzymes, modification of enzymes already present in the cells, or removal of inhibitors of these proteins. To address the question of the presence of soluble inhibitors, experiments were performed in which particulate fractions from undifferentiated, and granulocytic and monocytic mature HL-60 cells were mixed. If the undifferentiated cells contained a diffusable inhibitor of the kinase and phosphatase enzymes, then the combined activities of the mixtures would be less than

Figure 54. Tyrosine kinase activity of HL-60 cells treated with TPA and OAG. HL-60 cells were treated with 10^{-8} M TPA (○), 40 ug/ml OAG (△), or untreated (●), and tyrosine kinase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

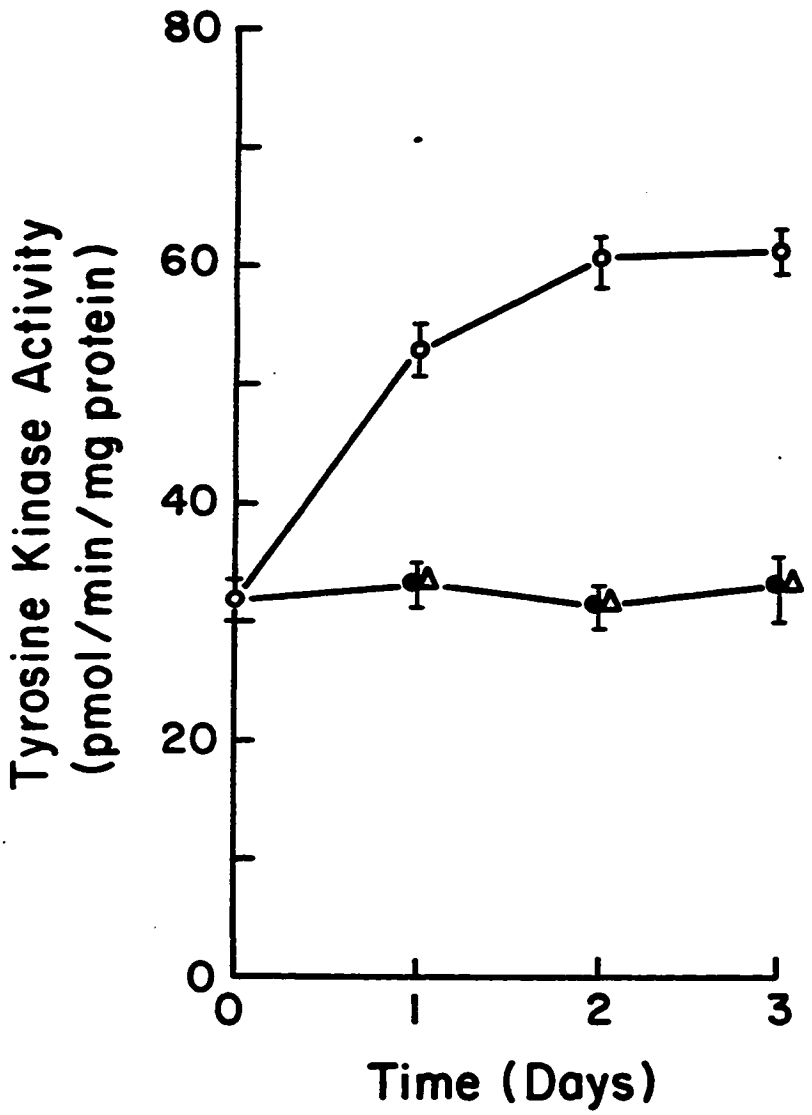


Figure 55. Phosphotyrosine phosphatase activity of HL-60 cells treated with TPA and OAG. HL-60 cells were treated with 10^{-8} M TPA (○), 40 ug/ml OAG (△), or untreated (●), and phosphotyrosine phosphatase activity was assessed. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.

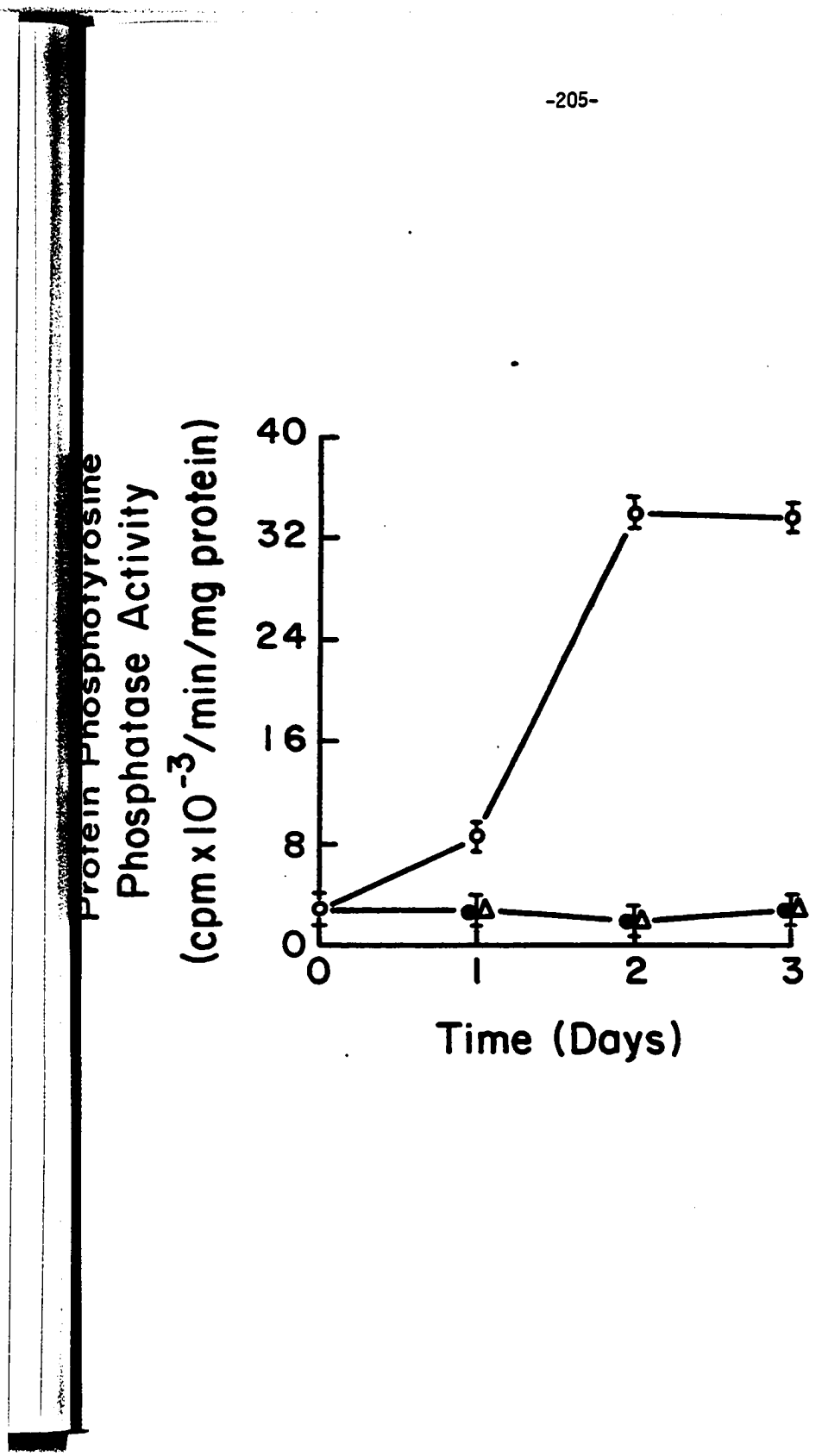
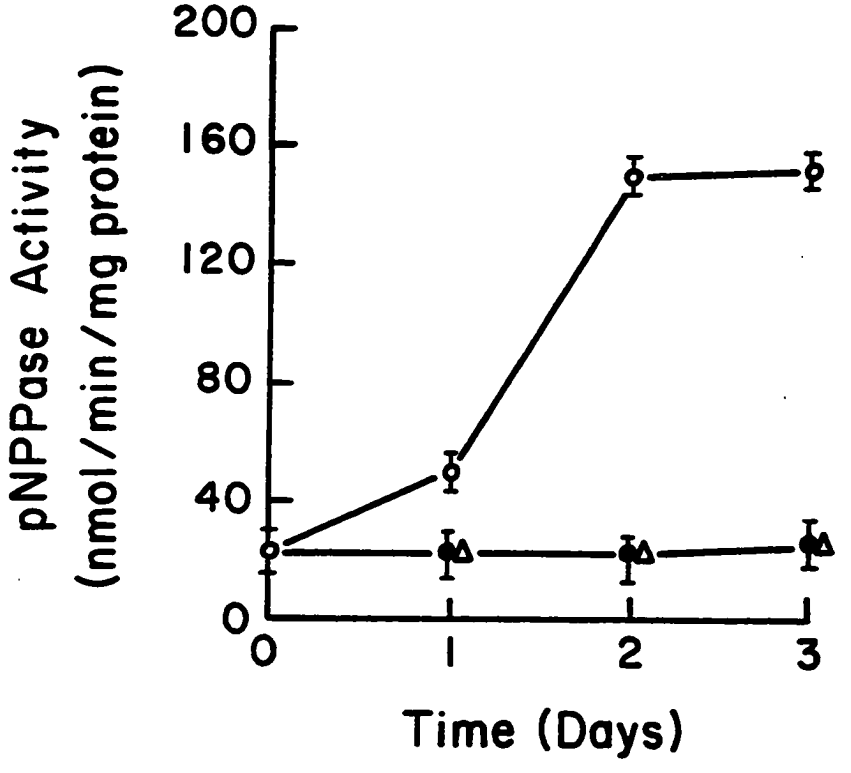


Figure 56. PNPPase activity of HL-60 cells treated with TPA and OAG. HL-60 cells were treated with 10^{-8} M TPA (○), 40 ug/ml OAG (△), or untreated (●), and PNPPase activity was measured. Each point represents the mean ± standard error of the mean of three experiments each done in duplicate.



additive. As shown in Table 16, all possible combinations showed additive activity, inidating that a reduced level of inhibitors with differentiation is an unlikely mechanism for the increased enzyme activities observed.

To further examine the relationship between the enzymes present before and after differentiation, the activities were compared for a number of biochemical properties. As shown in Table 17A the properties of tyrosine kinase activity in granulocytic and monocytic HL-60 cells were nearly identical to those of the untreated cells; similar results were found with the phosphatase activity (Table 17B).

Tyrosine kinase and protein phosphotyrosine phosphatase activities in mature peripheral blood granulocytes and monocytes. In trying to assess the importance of the changes in enzyme activities seen in differentiating HL-60 cells, a normal point of reference was needed for comparison. As HL-60 cells mature into granulocytes and monocytes, mature peripheral blood leukocytes were harvested for comparison. Using purification techniques described in the "Materials and Methods" section, pure populations of granulocytes and monocytes were prepared. As these cells have very short survival times in tissue culture (data not shown), it was impossible to label the cells long enough with [^{32}P] orthophosphate to measure phosphoaminoacid distributions. Particulate fractions could be prepared,

TABLE 16

Tyrosine Kinase and Phosphotyrosine Phosphatase Activities of Mixtures of Particulate Fractions

Treatment	Tyrosine kinase activity (pmol x 10^{-2}/min)	Phosphotyrosine phosphatase activity (cpm x 10^{-3}/min)
Untreated	74.6	3.4
DMSO	222.4	26.7
TPA	119.6	31.6
Mixtures:		
Untreated + DMSO	302.4	29.0
Untreated + TPA	197.3	36.8
DMSO + TPA	338.7	58.8

Particulate fraction protein (20 µg) from HL-60 cells that were untreated or induced to mature into granulocytes [1.2% (v/v) DMSO for 6 days] or into monocytes (10^{-8}M TPA for 3 days) were assayed for tyrosine kinase and phosphotyrosine phosphatase activity. Combinations of these fractions were also assessed for activity. Values represent the mean of two experiments; the range was less than 5% of the mean.

TABLE 17

Properties of HL-60 Tyrosine Kinase and Protein Phosphotyrosine Phosphatase Activities in Undifferentiated and Differentiated Cells

A. Tyrosine kinase:

	Untreated	Granulocytic	Monocytic
pH Optimum	6.4	6.4	6.4
Mn^{2+}/Mg^{2+}	1.8	2.0	2.1
GT/GAT	1.7	1.6	1.7
$37^{\circ}/23^{\circ}$	2.2	2.4	2.4

B. Phosphotyrosine phosphatase:

	Untreated	Granulocytic	Monocytic
pH Optimum	8.0	8.0	8.0
$37^{\circ}/23^{\circ}$	4.0	4.0	2.0
VO_4^{3-} IC_{50} ($\times 10^{-6}$M)	8.0	7.5	8.0
Thermal stability (IT_{50} at 37°C, hours)	5.0	5.0	4.5
Plasma membrane/ internal membranes	0.19	0.22	0.20

The properties of HL-60 tyrosine kinase and phosphotyrosine phosphatase activities were compared in untreated cells and those induced to mature along the granulocytic pathway [1.2% DMSO (v/v) for 6 days] or along the monocytic pathway (10^{-8}M TPA for 3 days). Mn^{2+}/Mg^{2+} represents the ratio of initial velocities using 12 mM Mn^{2+} and 15 mM Mg^{2+} (which were optimal concentrations for all 3 enzymes); GT/GAT represents the ratio of initial velocities using 1 mg/ml GT and 1 mg/ml GAT as substrates; $37^{\circ}/23^{\circ}$ represents the initial velocities at these two temperatures; plasma membrane/internal membranes represents the ratio of specific activities in these two fractions. VO_4^{3-} IC_{50} represents the concentration of VO_4^{3-} which inhibits 50% of the activity; and IT_{50} represents the time of exposure to 37°C at which 50% of the activity is inhibited.

however, and tyrosine kinase and phosphotyrosine phosphatase activities could be assessed. As shown in Table 18, peripheral blood granulocytes (predominantly neutrophils) have 60% of the tyrosine kinase activity and 3.4-fold more phosphotyrosine phosphatase activity then untreated HL-60 cells. This tyrosine kinase level is 21% that of HL-60 cells differentiated into granulocytes, and the phosphatase level is 47% as high. Monocytes contain 1.8-fold the tyrosine kinase activity of untreated HL-60, though these levels are comparable to those seen in monocytic-differentiated HL-60. The phosphatase level is 3.4 times greater than untreated HL-60, though only 37% of that of the TPA-treated cells.

Lack of tyrosine kinase and protein phosphotyrosine phosphatase activity in HL-60 conditioned medium. It has been shown that serum from patients with a number of malignancies contain elevated levels of tyrosine kinase activity, perhaps having been shed from tumor cells (71). Given this observation, experiments were conducted to determine whether either tyrosine kinase or phosphotyrosine phosphatase activity could be recovered from HL-60 conditioned medium. Medium was concentrated by diafiltration and tested for these enzyme activities. Neither fresh medium (RPMI 1640 supplemented with 10% (v/v) heat inactivated FBS) nor medium conditioned by untreated HL-60 cells, or those treated with DMSO or TPA contained any such detectable kinase or phosphatase activity (data not shown).

TABLE I8

Tyrosine Kinase and Protein Phosphotyrosine Phosphatase Activities in Peripheral Blood Granulocytes and Monocytes and HL-60 Cells

Cell type	Tyrosine kinase activity (pmol/min/mg)	Phosphotyrosine phosphatase activity (cpm x 10^{-3}/min/mg)
Peripheral blood granulocytes	23.8	12.4
Peripheral blood monocytes	71.0	12.1
HL-60, untreated	39.4	3.6
HL-60, granulocytic	114.6	26.4
HL-60, monocytic	72.8	32.8

Mature peripheral blood granulocytes and monocytes were isolated and purified as described in the text, and tyrosine kinase and phosphotyrosine phosphatase activities were measured. These were compared to those of HL-60 cells induced to mature along the granulocytic [1.2% DMSO (v/v) for 6 days] or monocytic (10^{-8}M TPA for 3 days) pathways. Values represent the mean of three separate experiments each done in duplicate; the range was less than 5% of the mean.

DISCUSSION

Although the initial events of carcinogenesis are almost certainly at the level of genomic DNA, evidence has accrued to indicate that epigenetic factors may modulate the transformed phenotype (89). It is probable that many types of neoplastic cells have a functional block in their ability to terminally differentiate, that is, to become mature non-dividing cells. The study of the differentiation pathway should thus shed light on the normal control of maturation, the development of neoplasms, and perhaps on mechanisms to reverse the transformation process. Differentiation entails two distinct events: the permanent cessation of proliferation and the acquisition of mature functions. In the consideration of tumor development the former is clearly of paramount importance. The HL-60 human promyelocytic leukemia cell line was chosen as a model of both leukemogenesis and bipotent differentiation. The present body of work examined the regulation of tyrosine phosphorylation -- a central mediator of proliferation (6-23) -- during the differentiation of these cells.

The initial observation that the intracellular content of phosphotyrosine decreased by an order of magnitude with the granulocytyic differentiation of HL-60 cells was consistent with expectations. In all (non-neural) systems examined, increased tyrosine phosphorylation correlates with

increased proliferation (15). It would thus be expected that terminal differentiation, the programmed shutdown of a cell's proliferative capacity, would be accompanied by a profound fall in phosphotyrosine residues.

The tyrosine kinase activity of these cells, through preliminary purification, was isolated as a single peak of activity on both molecular sieve and anion exchange chromatographies. Comparing the biochemical properties of this enzyme to a large number of other such kinases described in the literature (161-166) indicates that the HL-60 tyrosine kinase appears distinct (Table 19A). Given the relatively impure form of the enzyme, however, it is possible that other factors in the particulate fraction are distorting some of these properties. The fact that the kinase is membrane associated, predominately on the plasma membrane, and (at least in part) on the cytoplasmic face does not distinguish it from other known tyrosine kinases (161-166).

Much less is known about protein phsophotyrosine phosphatases. Nevertheless, the HL-60 activity, which also appears as a single peak on molecular sieve and anion exchange chromatographies, seems distinct from those phosphatases already characterized (Table 19B) (75, 136, 159). It will be interesting to see how these phosphotyrosine phosphatases compare with the classically described acid and alkaline phosphatases, with which they may have a good deal of homology (or identity) (79-81).

TABLE 19

Comparison of the Characteristics of HL-60 Tyrosine Kinase and Phosphotyrosine Phosphatase with Those of Previously Described Enzymes

A. Tyrosine kinase:

	HL-60	Insulin Receptor (162)	EGF Receptor (166)	IGF-1 Receptor (161)	Calf Thymus (165)	B Lymphocytes 163)
K_m (mM)	0.5-0.7	2.0	1.4			
Inhibition by NEM	++	+	++	--		
$[MG^{2+}]$ optimum (mM)	15	50	15	50	40	
$[Mn^{2+}]$ optimum (mM)	12	2	2	2		
Mn^{2+}/Mg^{2+}	1.5	2.0	2.8	0.1	2.0	
Stimulation by Zn^{2+}	+	--				
Inhibition by TLCK	+					--
Inhibition by 2 M NaCl	+				--	

B. Phosphotyrosine Phosphatase

	HL-60	CEF (75)	Calcineurin (159)	Rabbit kidney I	Rabbit kidney II (136)
pH Optimum	8.0	7.4		7.0-7.5	5.0
Inhibition by:					
VO_4^{-3}	+	+			
Zn^{2+}	+	+			
ATP, ADP	--	+			
divalent cations	--	--	+		
F^-	+	--			
Subcellular distribution (particulate/soluble)	20	0.42			

The characteristics of HL-60 tyrosine kinase and phosphotyrosine phosphatase were compared to those of enzymes reported in the literature. Mn^{2+}/Mg^{2+} represents the relative initial velocities using optimal concentrations of these two cations; subcellular distribution denotes the ratio of activities in the particulate and soluble fractions of the cells.

Knowing that the P-tyr content of HL-60 cells decreased with granulocytic differentiation, the activities of tyrosine kinase and phosphotyrosine phosphatase during this maturation was assessed next. In a seemingly paradoxical manner, tyrosine kinase activity increased in these cells as they matured, from 3- to 5-fold. (These changes are expressed on a per weight protein basis; as these cells undergo a reduction in protein content by 50% with differentiation, on a per cell basis this translates to a 1.5- to 2.5-fold increase in the kinase and a 3- to 4-fold increase in the phosphatase.) The temporal relationship between these changes in enzyme activities and functional maturation is consistent with a causal role. Differentiating myeloid cells, both normal and neoplastic, divide several times before undergoing terminal differentiation (147). It would thus be expected that changes in the regulation of tyrosine phosphorylation, if it is the final mediator of proliferation, would occur shortly before functional maturation rather than at the earliest "commitment" stages. It would be interesting to be able to follow the intracellular levels of phosphotyrosine during these intermediate stages of differentiation. The current methodology employing ^{32}P labeling, however, requires a long equilibration period (16 to 18 hours) in phosphate-free medium, thereby perturbing the system. As such, it is possible only to accurately quantitate phosphoaminoacid

levels in the untreated and terminally mature cells. Two techniques may be exploited to circumvent this problem. One involves the use of fluorescently labeled antibodies specific for P-tyr (46) which could be used to decorate cells *in situ*. Cellular fluorescence could then be quantitated by flow cytometry. A second method, currently being developed by this laboratory (in collaboration with Dr. Ian M. Armitage, Yale University) employs ^{31}P-NMR to quantitate cellular phosphoaminoacids. However, this technique currently suffers from insufficient sensitivity.

The increase in both kinase and phosphatase activities in conjunction with a fall in phosphotyrosine raises the question of what are the important changes involved. That these changes are specific for differentiation and not merely a byproduct of cytotoxicity or non-specific growth cessation is supported by the control systems employed. First, cells which stop proliferating due to nutrient/serum deprivation do not exhibit alterations in kinase and phosphatase activites. Second, the granulocytic differentiation induced by two different agents (RA and DMSO) cause similar changes. Third, the use of DMSO-resistant sublines demonstrated that the kinase and phosphatase changes do not result from a non-specific effect of DMSO, but occur only after differentiation has been triggered. Fourth, three anthracycline compounds were examined, all of which have similar structure and cause similar degrees of growth

inhibition. Only two of these agents, however, caused differentiation, and it was only those two which caused the changes in phosphotyrosine regulation. Fifth, an HL-60 subline which differentiated in the presence of TG was compared to the parent line which does not; using the same agent, at concentrations that cause similar inhibition of growth, only the cells which matured exhibited these changes. This regulation appears to exist in systems other than HL-60 cells, since the mouse myelomonocytic leukemia WEHI-3B underwent analogous changes. Finally, qualitatively similar changes occurred during the monocytic differentiation of HL-60 cells induced by TPA. OAG, an agent with similar biochemical effects, yet a non-inducer of differentiation, failed to cause these alterations.

One initial question is why does total phosphotyrosine fall in the presence of increasing tyrosine kinase activity. Several possible explanations exist. Given the greater increases in phosphotyrosine phosphatase activity, it could be that the phosphatase more than compensates for the kinase and thus lowers total phosphotyrosine. Such a mechanism would involve a futile cycle involving higher rates of tyrosine phosphorylation and still higher rates of dephosphorylation. Another possibility is that the kinase may be partitioned differently within the cell as has been suggested to be a means of regulation of the IGF-1 receptor (33); biochemical characterization and studies on subcellular

localization, however, have failed to find any such change. One may speculate that the decrease in total phsophotyrosine is due to a decrease in a substrate for tyrosine kinase. The critical substrates for tyrosine kinases have never been truly elucidated; the decrease in synthesis of such a substrate(s) could cause a fall in phosphotyrosine in the face of increasing tyrosine kinase activity. Ultimately, understanding the role of tyrosine phosphorylation will depend on the characterization of these substrates, and the functional changes caused by their phsophorylation. Phosphoaminoacid analysis by thin-layer electrophoresis cannot yield quantitative measurements, but can only present the ratio of P-tyr to P-ser to P-thr. Thus, although the proportion of P-tyr is decreasing, it is conceivable that the total quantity is actually increasing while the amounts of P-ser and P-thr are increasing even further. Such a situation seems unlikely as the total amount of phosphate incorporation into protein is decreased in differentiated cells (148). It has been found, though, that HL-60 cells induced to differentiate by DMSO or RA have increased levels of protein kinase C (148), a serine-threonine kinase. Cells induced to differentiate into monocytes by TPA showed a decrease in protein kinase C activity, but an increase in Ca^{2+}-, and phospholipid-independent phosphorylation of serine and threonine (148). Although phorbol esters exert at least some of their actions by activating protein kinase

C (111), TPA has been shown to both stimulate (149) and inhibit (150) various tyrosine kinases. The HL-60 enzyme is unaffected by this agent.

As mentioned above, a full understanding of the roles of the tyrosine kinases and phosphotyrosine phosphatases can only come with an understanding of the critical intracellular substrates that are modified. In actively proliferating tissue (6) and in undifferentiated HL-60 cells, phosphotyrosine represents little more than 1% of phosphorylated amino acid residues. As such a quantitatively small modification can have such profound effects on cellular function, it seemed clear that the tyrosine phosphorylation of certain critical substrates was central to the role of tyrosine phosphorylation.

The first intracellular substrates found for the tyrosine kinases were the enzymes themselves. The src gene product, for example, is phosphorylated on tyrosine (25). The functional significance of this autophosphorylation is unclear. Cross and Hanafusa (26) have used site directed mutagenesis to abolish the modified tyrosine, and found little difference between the mutant and wildtype proteins. Synder and Bishop (27), on the other hand, found decreased tumorigenicity in a similar mutant. Others have shown that incubating pp60 with non-hydrolyzable analogs of ATP and GTP led to increased tyrosine kinase activity, which suggested that nucleotide protection rather than phosphorylation per se

leads to increased kinase activity (28). Interestingly, these same workers found that the rate of autophosphorylation was independent of pp60 concentration indicating that the reaction was intra- rather than intermolecular. Bolen and coworkers (29) compared the kinase activity of pp60 isolated from two different tumors, and found that one of them was phosphorylated on tyrosine and possessed at least 10-fold greater kinase activity.

Several of the growth factor receptors have been shown to be autophosphorylated on tyrosine as well. The insulin receptor autophosphorylates in the presence of insulin, which leads to activation of the receptor. Once the receptor is phosphorylated and activated (with about a 10-fold increase in Vmax), the continued presence of insulin is no longer needed to maintain activation (30). Again, the reaction was shown to be intramolecular. The EGF receptor also phosphorylates itself, though no change in kinase activity or EGF binding affinity has been detected with phosphorylation (31). The PDGF receptor (32) and the IGF-1 receptor (33) are also phosphorylated on tyrosine following ligand binding, though the functional significance of these modifications have not been elucidated. Although the effect of autophosphorylation on kinase activity is unclear, it seems that this modification generally leads to increased enzyme activity. Thus, it may represent an amplification process, to further speed the transduction of a

proliferation signal. Nevertheless, for the diverse manifestations of the transformed state to be expressed, other cellular proteins must also be substrates for these enzymes. The fact that combinations of growth factors can lead to transformation just as transforming retroviruses can (34) suggests that common intracellular substrates may be targets for these enzymes.

Antibodies specific for phosphotyrosine have been developed and employed to examine cellular proteins which contain this modification. Marchisio et al. (35) used such polyclonal sera to examine RSV-transformed and untransformed fibroblasts by immunofluorescence. While the control cells had no significant staining with the antibodies, the transformed cells showed diffuse cytoplasmic staining and pronounced fluorescence at areas of cell-cell and cell-substratum contacts. The src product, as with most other tyrosine kinases, is membrane associated (in fact the ability to associate with membranes is critical for tranformation (36)), and thus it is not surprising that its substrates will be predominantly membrane-bound. Furthermore, many of the changes that occur with transformation involve the cell surface, including loss of contact inhibition, change in cell shape, and lack of substrate dependence. Thus, changes in the tyrosine phosphorylation of memberane-associated proteins was actively explored.

One cytoskeletal protein that has received a great deal of attention is vinculin. This 130,000-dalton protein is phosphorylated on tyrosine and associated with pp60 in RSV-transformed cells. Normally it is associated with adhesion plaques, regions where microfilaments terminate and cell-cell and cell-substratum junctions are formed. In transformed cells, the cytoskeleton becomes disordered, adhesion plaques decrease in number, and cells assume a more-rounded shape (37). Viculin thus seemed an excellent example of a critical tyrosine kinase substrate. Numerous analyses, however, have failed to show any correlation between vinculin phosphorylation on tyrosine and changes in intracellular microfilament structures (37). As such, the relationship between tyrosine phosphorylation and cell surface changes remains obscure. An interesting observation confirms the likely importance of tyrosine phosphorylation and at least some cell surface phenomena. The ability of tumor cells to degrade extracellular fibronectin is important in invasion and metastasis. With the use of dual labeling techniques, it has been shown that areas of the cell surface at which fibronectin degradation is occurring correspond to regions at which pp60 localize (38).

Another well known change that occurs with transformation is an increase in the rate of glycolysis, the so-called Warburg effect (39). Thus Cooper and coworkers examined changes in tyrosine phosphorylation of glycolytic

enzymes in RSV-transformed cells (40). They found that three of the enzymes they examined became phosphorylated on tyrosine during transformation: phosphoglycerate mutase, lactate dehydrogenase, and enolase. Although it is not known at this time how such modifications affect the intracellular function of these enzymes, there is reason to question the significance of these changes. These include the findings that: the stoichimetry of phosphorylation is low (less than 5%); the activities of these enzymes does not seem to be altered by transformation; and the rate of glycolysis is increased in other types of transformed cells which do not contain these modifications (40). Thus, the importance of the phosphorylation of glycolytic enzymes, though intriguing, remains unclear.

As topoisomerases are critical enzymes in DNA replication, and as these enzymes interact with DNA via a tyrosyl-phosphate link, Tse-Dinh et al. (41) examined the effect of a viral and a eukaryotic cellular tyrosine kinase on a bacterial topoisomerase in a cell-free system. The authors found that both kinases could phosphorylate the topoisomerase on tyrosine residues and that such phosphorylation led to a 10-fold decrease in the ability to relax supercoiled plasmid DNA. This was the first demonstration of a change in enzyme activity resulting from tyrosine phosphorylation (other than changes following autophosphorylation). It is clear that enzymes such as

topoisomerses which affect the physical structure of DNA are important in gene expression and DNA replication, and hence they are attractive putative substrates for tyrosine kinases. There is no evidence, however, that such modifications occur in vivo; furthermore, nearly all of the tyrosine kinases described thus far are extra-nuclear and membrane bound. Thus one would have to postulate several steps to explain the transfer of a membrane protein to the nucleus.

Another attractive cellular substrate for tyrosine kinases is the S6 kinase. Phosphorylation of the 40S ribosomal protein S6 seems to be closely associated with the initiation of protein synthesis, and such phosphorylation is rapidly induced by mitogenic agents, tumor promoters, and RSV (42). The site of action of all of these agents appears to be a serine kinase which phosphorylates S6. Although it has yet to be shown that the tyrosine phosphorylation of the S6 kinase increases its activity, the data reported thus far are very suggestive for such a mechanism. As such it represents perhaps the first cellular substrate for a tyrosine kinase whose activity is modulated by such phosphorylation.

Other protein substrates for tyrosine kinases have been described. The phospholipase inhibitory protein lipomodulin has been shown to be phosphorylated on tyrosine following mitogen stimulation of murine thymocytes (43). An

endogenous tyrosine kinase can phosphorylate the nicotinic acetylcholine receptor in Torpedo californica (44) and calmodulin can induce the tyrosine phosphorylation of the 17-Beta-estradiol receptor (45). The functional significance of any of these modifications is unclear.

Two techniques have come into use for the identification of phosphotyrosine containing extracts. One involves the use of antibodies specific for phosphotyrosine (46). Cells can be labeled with ^{32}P-orthophosphate, and proteins containing phosphorylated tyrosine residues immunoprecipitated with such a reagent. Alternatively, after ^{32}P-labeling, cellular proteins can be separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel can then be treated with alkali, which preferentially cleaves phosphoserine and phosphothreonine bonds, leaving the phosphotyrosine containing proteins still labeled (47). Such proteins can then be visualized and isolated from the gel. Using such techniques, a number of proteins have been found that are phosphorylated on tyrosine under a variety of conditions; the functions of these proteins are unknown (48-51).

In studying tyrosine kinases, a number of exogenous peptide substrates have been used. These include angiotensin (52), gastrin, ACTH (53), random polymers of glutamic acid, alanine, and tyrosine (54), and the src peptide (the sequence of pp60 surrounding the tyrosine that

is autophosphorylated) (53). These peptides have in common the presence of tyrosine as the only hydroxylated amino acid and thus all phosphate incorporation represents tyrosine kinase activity. The diversity of these substrates in size and charge indicates that at least *in vitro* these enzymes appear to have a wide specificity.

The evidence for the importance of tyrosine kinases in transformation led many researchers to examine potential critical intracellular protein substrates for these enzymes. In 1984, however, work by Sugimoto et al. (55) added a new dimension to this search. These authors found that in addition to being a protein kinase, pp60 could also phosphorylate phosphatidylinositol to the 4-phosphate and 4,5-bisphosphate. Certain membrane signals can cleave these molecules into diacylglycerol and inositol 1,4-bisphospate and inositol 1,4,5-trisphosphate, respectively. Diacylglycerol stimulates the calcium- and phospholipid-dependent protein kinase C, while the inositol phosphates, particulary the trisphosphate, can mobilize calcium from intracellular stores. One of the key steps in this signal transduction sequence is the intitial phosphorylation of phosphatidylinositol, the one apparently calalyzed by the putative tyrosine kinase. At this point it was suggested that the difficulty in discerning appropriate intracellular protein substrates for the tyrosine kinases was because the actual substrate was in fact the phosphatidylinositol.

Over the last few years, however, evidence has accrued suggesting that tyrosine kinases are in fact distinct from the phosphatidylinositol kinases. The laboratory that first noted the lipid phosphorylation employed thermal inactivation to demonstrate that the phosphatidylinositol kinase protein was in fact distinct from the tyrosine kinase of pp60 (55). At the same time, it was shown that phosphatidylinositol kinase could be separated from the tyrosine kinase of the EGF receptor (56) and of two transformed cell lines (57). Although these two pathways seem closely related, it now seems clear that they represent distinct systems The critical substrates for tyrosine phosphorylation in HL-60 have yet to be identified. It has been shown in the present work that phosphatidylinositol does not compete with phosphorylation of the peptide substrates, suggesting that protein phosphorylation is the central process. Employing the techniques described above, it may be possible to dissect the important substrates for tyrosine phosphorylation in HL-60, which will be important for completely understanding this system.

The evidence is very strong for the importance of tyrosine phosphorylation in regulating growth control, and this modification has been conserved evolutionarily, having been found in yeast (58) and photosynthetic bacteria (59). The particular significance of tyrosine phosphorylation versus serine or threonine phosphorylation is obscure.

Modification of tyrosine may allow the selective modulation of critical enzymes which contain tyrosine hydroxyl groups at their active sites such as topoisomerases (41) and ribonucleotide reductase (60). Alternately, the phosphorylation of tyrosine may disrupt the structural stabilization effect of aromatic-aromatic interactions (61), allowing this modification to cause gross conformational changes in critical substrates. As notes above, however, correlations between tyrosine phosphorylation and functional changes have been weak.

Other kinases are also known to change with HL-60 maturation. The insulin receptor, for example, increases in expression during monocytic differentiation of HL-60 cells (151); during granulocytic maturation, by contrast, it decreases (151). The GM-CSF receptor, another tyrosine kinase, increases in activity with monocytic differentiation, but does not change with granulocytic differentiation (152). It thus seems unlikely that either of these receptors accounts for the increased tyrosine kinase activity which accompanies granulocytic ormonocytic differentiation of HL-60 cells.

Much less is known about changes in specific phosphatases with differentiation, though an increase in acid phosphatase activity with HL-60 granulocytic maturation has been reported (99). The relationship between this phosphatase and that described in the present work is

unclear. The phosphotyrosine phosphatase reported here does have activity at pH 4 (where the acid phosphatase activity was measured), and thus they may be the same activities.

To try to determine how the changes which attend HL-60 differentiation are related to normal maturation, the levels of tyrosine kinase and phosphotyrosine phosphatase activities were assessed in normal peripheral blood granulocytes and monocytes. As these cells survive very poorly in tissue culture, it proved impossible to successfully label them for 16 to 18 hr with ^{32}P for phosphoaminoacid analysis. (These cells would thus be especially suitable for the non-radioactive methods of phosphotyrosine determination described above.) Mature granulocytes contain 60% the tyrosine kinase activity of untreated HL-60 cells (Table 18), and 21% that of HL-60 cells induced along the granulocytic pathway. They also contain 3.4-times the phosphotyrosine phosphatase of immature HL-60 cells, but only 47% that of differentiated ones. Thus, mature granulocytes possess very low tyrosine kinase activity, but relatively high phosphotyrosien phosphatase activity. It is likely that the enzyme activities of HL-60 cells reflect that of promyelocytes, the normal progenitor cell in the bone marrow from which they arose. It would be very difficult to experimentally verify this, as such cells would be hard to isolate and purify in sufficient quantity from normal bone marrow. Compared to

normal mature granulocytes, these cells have higher tyrosine kinase and lower phosphotyrosine phosphatase levels. They would also be expected to have higher phosphotyrosine levels, given this enzyme distribution, which would be consistent with the fact that promyelocytes are proliferating cells and granulocytes are end-stage non-dividing cells. Thus, as these cells differentiate, one may postulate that they decrease their phosphotyrosine levels (as they cease to proliferate) by a coordinated decrease in tyrosine kinase and increase in phosphotyrosine phosphatase activities. Such coordinated regulation would contain a safety mechanism, as is common in similar biological systems, to prevent uncontrolled proliferation. The failure of the kinase to decrease in activity would be compensated by the increase in phosphatase activity; likewise, a failure of the phosphatase to increase in activity would not be deleterious if the kinase activity decreased. Uncontrolled proliferation would only result from the failure of both arms of the mechanism, as apparently has occurred in the HL-60 leukemia. If this scenario is correct, then an agent which induces the terminal maturation of HL-60 cells could overcome this block by either decreasing kinase activity, increasing phosphatase activity, or both. From the data presented in this work, it seems that all of the inducers of granulocytic differentiation only increased the phosphatase activity, to a level higher than that present even in normal

granulocytes. The tyrosine kinase activity actually incrased somewhat (though on a per cell basis this increase was small). Given this, one may postulate that in the differentiation process, the tyrosine kinase activity fails to decrease as is normal (this may be due to the genetic lesion responsible for the leukemia, as discussed below). The agents which induce differentiation, however, are able to generate such a large increase in the phosphatase activity that the elevated kinase activity is overcome, phosphotyrosine levels fall, and terminal maturation occurs.

One question which arises is why does the kinase activity fail to decline with differentiation. It is known that when HL-60 cells mature, their mutant N-ras gene continues to be expressed as before (114,115). Although the ras gene product p21 is believed not to possess any tyrosine kinase activity (116), it could conceivably activate a normal cellular tyrosine kinase. There is precedence for such a mechanism in the transformation pathway of the polyoma virus. This virus must have an active protein, the so-called middle T antigen, to effect transformation (153). It had been believed that this viral product possessed tyrosine kinase activity; recently, however, it was found that this protein interacted with a normal cellular tyrosine kinase, thereby increasing its kinase activity (153). Although a similar mechanism has never been defined for the ras product, work in this

laboratory has shown that the transfer of the activated ras oncogene from HL-60 cells to NIH/3T3 fibroblasts causes an increase in tyrosine kinase activity of the recipient cells (Frank, D. A. and Sartorelli, A. C., unpublished observations).

Such a theory could account for the elevated tyrosine kinase activity of the differentiated cells, and would explain why such an elevated level of the phosphatase activity is necessary. It is known that mature HL-60 cells are not functionally identical to normal granulocytes. They generally do not reach the ultimate morphological stage of maturity and they lack some of the most specialized enzyme activities (112). Such defects may reflect the abnormality in the tyrosine phosphorylation regulatory mechanisms.

Monocytes from peripheral blood contain 80% higher tyrosine kinase activity than untreated HL-60 cells, about the same as that of HL-60 cells induced to differentiate along the monocytic pathway (Table 18). They possess 3.4-times the phosphotyrosine phosphatase activity as the immature cells, yet only 37% that of the monocytically induced cells. Thus monocytic differentiation of HL-60 differs from granulocytic, in that the tyrosine kinase activity of the monocytes is elevated. The functional implications of this are unclear; it could reflect the elevated levels of the receptor for GM-CSF (a tyrosine kinase) found in mature monocytes, as noted above (152).

Alternatively, it could represent the activity of a non-proliferative tyrosine kinase pathway, as found in the nervous system (29). The ability to determine the phosphotryrosine content of these cells would be a major aid in understanding this system, as discussed above.

Although the regulation of phosphotyrosine by coordinated kinase and phosphatase activities is an attractive hypothesis, there is little direct evidence to support it. If the increased phosphatase activity is critical to differentiation, one would expect that an agent which inhibited this activity would prevent differentiation. In preliminary experiments, orthovanadate, a potent inhibitor of the phosphatase, failed to show profound inhibition of HL-60 differentiation (Frank, D. A. and Sartorelli, A. C., work in progress). In a similar manner, vanadate has been shown to cause transformation of fibroblasts with increased phosphotyrosine levels, as it inhibited phosphatase activity (80). It would also be interesting to observe how agents which affected the kinase, such as quercetin, influenced cellular phosphotyrosine levels and HL-60 differentiation. This also touches on the issue of the relationship of cessation of proliferation and development of the mature phenotype. Would an agent that lowered phosphotyrosine levels merely stop proliferation, or would it also trigger maturation? If a tyrosine kinase were introduced into HL-60 cells by a retroviral vector, would

still higher levels of the phosphatase be needed for differentiation? Would the microinjection of a phosphatase stop proliferation and/or induce differentiation? All of these experiments are becoming technically feasible, and would more directly address the biological role of this system.

Although a great deal of attention has been paid to the tyrosine kinases as the dominant oncogenes responsible for transformation, much less has been done to elucidate the rolse of the countervening phosphatases. This work highlights their possible importance in cellular growth control. As noted in the "Introduction," these proteins serve as excellent candidates for the products of the so-called recessive oncogenes. Such oncogenes believed to be active in a number of hereditary cancers such as Wilm's tumor and retinoblastoma, are apparently activated when both alleles are functionally deleted (85,86). In other words, the loss of its product results in transformation (in contrast to the dominant oncogenes in which the presence of the product causes the malignancy). As discussed above, the loss of phosphotyrosine phosphatase activity could lead to the unbalanced accumulation of phosphotyrosine even in the presence of normal levels of tyrosine kinase. It would be of interest to examine tissue from these tumors and compare the phosphotyrosine, tyrosine kinase, and phosphotyrosine phosphatase levels to those of the normal surrounding

tissue. If this hypothesis is true, one would expect to see elevated phosphotyrosine and decreased phosphatase activities in the tumor.

In a similar vein, senescent human fibroblasts have been found to have large amounts of an mRNA species which can inhibit proliferation when introduced into dividing cells (154). Again, phosphotyrosine phosphatase would be an attractive (and testable) product of these messages.

It is also known that RSV cannot cause tumors in avian embryos (155). While the mechanism of this resistance is unknown, one could hpyothesize that the rapidly dividing embryo cells already contain high levels of tyrosine kinase and appropriate levels of phosphotyrosine phosphatase to maintain growth control. In this setting, the added tyrosine kinase activity of the RSV kinase (pp60) may not be able to effect transformation.

This work and that of others would suggest that the phosphotyrosine system is an attractive target for chemotherapeutic intervention. Tyrosine kinases would certainly be one group of enzymes whose inhibition might cause potent anti-neoplastic effects. In addition, compounds which activate phosphatase activity might also find use in the oncologist's armamentarium. The HL-60 system described in the present work may well be an appropriate model system for exploring these possibilities.

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